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For Host Factors Weddings and a Koch's Bacillus Funeral: Actin, Lipids, Phagosome Maturation and Inflammasome Activation

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1. Introduction

When a particle or microorganism enters the host, is readily phagocytosed by the local macrophages and dendritic cells. If the microorganism is a non-pathogenic bacteria such as *Mycobacterium smegmatis*, the bacteria containing phagosomes will fully mature. Maturation refers to a multi-dimensional and complex process that integrates biochemical alterations on the phagosomal membranes, resulting as a direct response from the external stimuli and indirectly from the immediate response at the gene expression level in the form of transcriptional programs, as well as from its regulation. This, in turn, leads to modifications at the intracellular architecture, with the reorganization of the organelle dynamics, dependent on the assembly of actin, and resulting ultimately in the fusion of phagosomes with lysosomes. Not only several biochemical pathways have been implicated, but also physical and mechanical events will determine the faith of the bacteria containing phagosome. At the biochemical level, acidic hydrolases and the proton ATPase will reach the phagosomes while maturing and promote the lowering of the intraphagosomal pH, thereafter contributing to the killing of the bacteria. The production of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS) and the reactive oxygen species (ROS) by phagosome oxidases (phox) is also known to be important for the bacterial killing. NO and cytokines are an important part of the inflammatory response which is downstream of transcription factors such as nuclear factor-κB (NF- κB).

When the pathogenic *Mycobacterium tuberculosis* enters the macrophage phagosome, the maturation process is blocked. We have shown that at least three distinct processes are targeted: actin assembly, fusion with lysosomes and acidification (Anes et al., 2003, Anes et al., 2006, Castandet et al., 2005). It has been suggested that specific *M. tuberculosis* lipids such as the sulfolipids (Goren et al., 1976), and phenolic glicolipids (Rhoades et al., 2003), and secreted peptides (Walburger et al., 2004) have been implicated as effectors involved in blocking the phagosome maturation to phagolysosome. These factors, which have been reported to be transferred to different host membranes in infected cells, are likely to be the cause of the well-described inhibition of pro-inflammatory responses, including the inhibition of TNF- α secretion, and the restriction of phagosome motility.

These blocking mechanisms, which are still yet poorly understood, can somehow, be reversed. Our published data show that phagosome maturation is directly linked to actin assembly on membranes (Defacque et al., 2000). This will consequently lead the organelles to aggregate before they fuse with lysosomes (Jahraus et al., 2001). We also described the addition of several pro-inflammatory lipids to *M. tuberculosis* infected cells with surprisingly strong killing effects (Anes et al., 2003, Gutierrez et al., 2009, Jordao et al., 2008). These selected lipids can manipulate the phagolysosome maturation by interfering with the actin machinery. In addition to these signaling lipids, ATP, the P2X7 receptor and cAMP were shown to be involved in actin assembly and the killing/survival of pathogenic mycobacteria (Kalamidas et al., 2006, Kuehnel et al., 2009, Kuehnel et al., 2009b). Furthermore, some lipid effectors for actin assembly also control NF-kB (Gutierrez et al., 2009). Using microarray analysis, we linked NF-kB in the regulation of many lysosomal enzymes and membrane-trafficking regulators, including cathepsins, LAMP-2 and Rab34, during infection (Gutierrez et al., 2008).

Important classes of lipids that are effectors for actin assembly and for NF-kB control include eicosanoids. Eicosanoides produced from arachidonic acid such lipoxin X4 (LX4) as well ATP, and the P2X7 R are all additionally involved in a necrotic-programmed cell death (Chen et al., 2008). Distinct molecules were found to play opposite roles either inducing apoptosis or necrosis during infection. In fact while virulent *M. tuberculosis* promotes necrotic cell death and inhibits apoptosis, the non-virulent strain H37Ra induces apoptosis that results in lower bacterial viability. Pyroptosis in the context of tuberculosis is controversial: from one side it leads to potent pro-inflammation that drives tuberculosis; from other side it allows the release of intracellular bacteria and their escape to hydrolytic digestion. This type of programmed cell death depends on the assembly of a specific inflammasome leading to caspase 1 activation and IL-1β and IL-18 secretion. Some groups claimed that *M. tuberculosis* might block the inflammasome activation (Master et al., 2008). We have shown that *M. tuberculosis* in human macrophages, not only activates caspase 1 and IL1-β secretion but also that different inflammasomes are assembled during infection (Mishra et al., 2010).

So we propose in this chapter to present an overview of host factors that may be manipulated in order to reverse phagosome maturation arrest, NF-kB translocation, inflammasome activation and therefore boosting the macrophage killing abilities to Koch's bacilli.

2. The disease

Tuberculosis is usually a lung infection caused by inhalation of Koch's bacilli within microdroplets, but it can affect any organ. In the lung, bacilli are envisaged to be engulfed by cell patrolling the alveolar surface such as alveolar macrophages and tissue dendritic cells (DC). These cells transport the pathogens to the lung interstitium and draining lymph nodes. At both sites, primary lesions develop that rarely cause disease. These professional phagocytes that are devoted to maintain the lungs cleaned from particles, produce a series of bacterial insults designed to kill pathogens. However *M. tuberculosis* developed capacities to subvert the killing mechanisms of phagocytes to allow their intracellular survival and/or

replication. The first contact with these phagocytic cells will display a series of innate immune mechanisms that may lead to the complete clearance of the bacilli. These include the release of NO, ROS, the fusion of the phagosome with the lysosome with the simultaneously bacteria digestion by acidic hydrolases and antimicrobial peptides (Cooper et al., 2000, Liu et al., 2007, MacMicking et al., 1997). Ultimately the programmed cell death of the infected host cell will kill the reminiscent infecting bacteria (Park et al., 2006).

Furthermore the initially infected cells release pro-inflammatory cytokines which leads to recruitment of more DC, monocytes and neutrophils from the blood stream and the infected DC become mature and migrate to the local lymph node where they activate specific T cells. The cytokines IL-12 and IL-18 from the infected cells induce natural killer cell (NK) activity, and the NK cells in turn produce IFN-γ, which activates the macrophages to produce TNF-α and more microbicidal effectors (Korbel et al., 2008). In fact resting phagocytic cells are designed to uptake and clear bacteria by phagolysosome fusion with full bacteria digestion, while activated macrophages are reprogrammed to produce more free radicals, and decrease the extend of bacteria digestion in order to produce antigens for immune cross presentation. Through cytokine and chemokine signaling, other immune cells are recruited and the pathological hallmark of TB, the granuloma, is formed. In the granuloma, macrophages differentiate further into epitheloid cells or foamy macrophages, or fuse to form giant cells, and become surrounded by lymphocytes and an outer cuff of fibroblasts and extracellular matrix proteins. At this stage infected people may complaint for symptoms that may resemble a simple cold.

Thereby, the bacilli are contained until the granuloma fails due to immunosuppression (Russell, 2007). For a long time, the granuloma was viewed as beneficial only for the host – it coincided with the onset of adaptive immunity and reduction of bacterial growth in the lung – but recent studies in zebrafish embryos infected with a close relative of *M. tuberculosis*, *Mycobacterium marinum*, have indicated that mycobacteria also use the granuloma for their benefit upon initial infection, recruiting new macrophages to allow spread between host cells (Davis & Ramakrishnan, 2009). This stage were the centre of the granuloma is formed by infected macrophages usually is referred as a latent infection without disease symptoms and according to the World Health Organization (WHO reports 2008) corresponds to one third of the world infected people. From these only 5-10% will develop disease during their lifetime (Kaufmann & Parida, 2007).

When the granuloma centre becomes caseous containing necrotic macrophages following necrotic programme cell death, latent infection becomes in active disease, which in advanced TB form cavities in the lung. Historical texts identify the disease as "consumption," "wasting away," "king's evil," "lupus vulgaris," "the white plague" or "phthisis" based on its clinical manifestations (Donoghue, 2009). Although mycobacteria are able to survive and proliferate within phagocytes, it should be noted that during active pulmonary tuberculosis they are preferentially found in the extracellular space. The necrotic centers of infection sites during active disease contain liquefied, so-called caseating, debris. These lesions are rich in cellular detritus, membrane phospholipids and cholesterol, which serve as an important carbon source for mycobacteria. Spillage of infectious bacilli into the airways occurs when the structure ruptures, and this allows spread to new individuals (Russell, 2007).

The present overview of host innate immune response to *M. tuberculosis* highlight the relevance of the phagolysosome fusion, host programmed cell death and inflammation as important mechanisms whose possibility of control by molecular effectors may provide alternative therapies for TB.

3. Phagosome maturation blockade into phagolysosome by *M. tuberculosis*: targeting phagosomal actin assembly, fusion and acidification

Phagocytosis — the engulfment of particles by unicellular organisms and higher eukaryotic cells — was first observed and documented by Metchnikoff around the beginning of the last century. His observations with macrophages ("big eater" in Greek) persuaded him that these professional phagocytes were the primary defence against microbial invaders. Although this view garnered little support at the time, the central role of phagocytes in immune protection is now universally accepted. The successful completion of these protective activities requires maturation of the phagosome into an acidic, hydrolytically competent, organelle that can fuse with lysosomes, which contain a battery of degradative enzymes. In fact we were able to show that pH acidification renders mycobacteria more susceptible to kill with the highest susceptibility observed in BCG at a pH bellow 6.3 when compared to *M. bovis* (Jordao et al., 2008).

Upon uptake of a pathogen by receptor-mediated phagocytosis into a macrophage, the resulting phagosome undergoes a series of fusion and fission events with the endocytic pathway. One of the important functional characteristics of the maturation process is that newly formed phagosomes, like early endosomes, mature to a state where they no longer fuse with early (or maturing) endosomes; only then can they fuse with late endosomes and lysosomes (Jahraus et al., 1998).

Two theories of how the communication between the phagosome and the endosomal network and lysosomes occurs have been postulated – the kiss and run-hypothesis where the vesicles interact through transient fusion and fission events via a fusion-pore-like structure (Desjardins, 1995), and the fusion hypothesis where the phagosome completely fuses with pre-existing endosomes (Flannagan et al., 2009). It has been argued that the kiss and run-hypothesis is more probable since phagosomes do not acquire endosomal proteins and solutes simultaneously, and since molecules of different size are recruited from the same endosomal type at different time points (Tjelle et al., 2000). In addition to acquisition through interaction with endosomes and lysosomes, phagosomes can acquire proteins and lysosomal hydrolases from the trans Golgi network (Rohde et al., 2007).

The ageing process of phagosomes is tightly regulated, although several pathogens have the capacity to subvert this process. During the late 1960s and early 1970s, D'Arcy Hart reported that pathogenic mycobacteria, including *M. tuberculosis*, are maintained in vacuoles that are not accessible to tracers known to be delivered to lysosomes, and that this 'non-fusogenic' phenotype correlates with the viability of the infecting organisms (Armstrong & Hart, 1975, Hart & Armstrong, 1974, Hart et al., 1972). These data marked one of the earliest applications of modern cell biological techniques to an intracellular bacterial infection. Indeed these seminal studies brought cell biology and microbiology together.

The demonstration that vacuoles containing *M. tuberculosis* contain both transferrin receptor and major histocompatibility complex (MHC) class II molecules indicates that they communicate with the cell plasma membrane (Clemens & Horwitz, 1995, Clemens & Horwitz, 1996). Although these vacuoles fail to fuse with lysosomes, they remain fusion-competent, acquire some 'lysosomal' proteins from the synthetic pathway of the host cell, and undergo fusion with other vesicles of the early endosomal system. The consensus that has emerged in the literature is that pathogenic mycobacteria have evolved a strategy to arrest the normal maturation process of phagosomes after uptake by macrophages. Because of the paucity of the vesicular proton ATPase on the membrane of mycobacterial phagosomes, *M. avium* and *M. tuberculosis* phagosomes fail to acidify in the way that phagosomes with inert particles do (pH 4.5-5) and maintain a pH of around 6.3 (Sturgill-Koszycki et al., 1994). In addition mycobacteria express a urease generating ammonia, which could buffer the lumen to counteract proton influx (Gordon et al., 1980). Moreover, mycobacterial phagosomes carry only limited amounts of lysosome-associated membrane proteins such as LAMP1, and have limited hydrolytic enzyme activity such as cathepsin B/L. Cathepsin D, which requires proteolytic and auto catalytic processing at low pH lysosomes to generate the enzymatically active form, is only present in its high molecular weight pro-form in mycobacterial phagosomes (Sturgill-Koszycki et al., 1996). This indicates that mycobacterial phagosomes are restricted from fusion events with downstream late endosomal/lysosomal compartments.

Once an appreciation of the physiology of these vacuoles was attained, groups started to probe for proteins known to regulate the membrane fusion events associated with endosome–phagosome maturation. It is well established that small GTPases of the Rab family are present on certain organelles and take part in specific fusion events. For example, Rab5, which functions during early endosome homotypic fusion, is normally recruited to nascent phagosomes and dissociates from these vesicles as they acquire Rab7, another GTPase that functions during fusion of late endosomes and lysosomes. Rab5 is associated with, and retained by, phagosomes containing live *Mycobacterium bovis* (bacillus Calmette-Guérin or BCG) or *M. tuberculosis* (Via et al., 1997). Rab5 is associated with phagosomes immediately after phagocytosis and facilitates the recruitment of Rab5 effector proteins, EEA1 and class III phosphatidylinositol-3-phosphate kinase which drives the synthesis of the early endosomal lipid phosphatidylinositol 3-phosphate (PI(3)P) (Vieira et al., 2001). PI3P interacts with Rab5 and EEA1 to promote endosome maturation. Membrane bound Rab5 is rapidly dissociated from the phagosome after its activation. Rab7 appears on the phagosome membrane after Rab5 dissociation and resides on the membrane during phagosome maturation (Vieira et al., 2003). After acquisition of Rab7, phagolysosome biogenesis is accelerated by the recruitment of Rab7-interacting-lysosomal-protein (RILP) to the phagosome (Harrison et al., 2003). Recently it was show that more Rab GTPases are recruited during progression of phagosome maturation and that their release and/or dissociation from *M. tuberculosis*-containing phagosomes has the relevance for the *M. tuberculosis*-induced inhibition of phagolysosome biogenesis. (Seto et al., 2011). Finally, the early endosomal v-SNARE cellubrevin is degraded on mycobacterial phagosomes, making it unavailable for fusion events and delivery of transport vesicles from the trans-Golgi (Fratti et al., 2002).

Rab GTPases also mediate a microtubule-based transport system that is likely to be responsible for providing the scaffold for endosome movement . Microtubules together with actin cytoskeleton are important for phagosome processing, for recycling from the phagosome and for phagosome intracellular transport and fusion with endocytic organelles (Southwick et al., 2003). In fact during maturation phagosomes move from the cell periphery to the perinuclear region where phagolysosome fusion occurs (Talaat et al., 2004).

Manipulation of Rab and SNARE proteins is a key component of the survival strategy of *Mycobacterium* (Kumar et al., 2010). Moreover, expression of Rabs is down-regulated in patients with tuberculosis compared with healthy *M. tuberculosis*-infected donors (Jacobsen et al., 2005). We found that the synthesis of Rab34 was elevated in a NF-κB dependent manner in both *M. smegmatis*- and *M. avium*-infected macrophages. However, the regulation of this protein was the opposite in the two systems. In *M. smegmatis*-infected cells, NF- κB was a positive regulator, whereas in *M. avium*-infected cells it behaved as a negative regulator. Although the mechanism of this difference is open, these data suggest that *M. avium* has the ability to switch the transcriptional response linked to NF-κB to a state more conducive for its survival (Gutierrez et al., 2008). Rab34 has been shown to regulate lysosome positioning within the cell (Wang & Hong, 2002). Since lysosome positioning may be required for mycobacterial killing, we are testing the hypothesis that the regulation of Rab34 expression controls the ability of phagosomes to be positioned close to lysosomes.

In addition to microtubules several studies have shown that actin filaments increase the uptake of ligands and their delivery to the degradative compartments downstream of the region where microtubules are required (Durrbach et al., 1996). The actin filaments also are involved in the motility and distribution of early endosomes via Rho D protein (Murphy et al., 1996). However, to what extent the actin filament network is required for interaction of phagosomes with early and/or late organelles of the endocytic pathway?

It is clear that manipulation of actin's fate by intracellular pathogens within the host cytoplasm is a key event that determines bacterial survival. The roles of actin in host-microorganism interactions can be used to categorize pathogens into four groups: 1) some use actin to enter non phagocytic cells; 2) some use it to avoid uptake by phagocytic cells; 3) some use it to promote attachment to the host plasma membrane forming pedestals; 4) some escape from the phagosome and use the actin to move within cells and travel from cell to cell. In addition to the pathogens that have an extraphagosomal way of life, it is clear that some pathogens can modify the actin cytoskeleton from within the phagosome.

Pioneer work by de Chastellier and co-workers indicates that intracellular *Mycobacterium avium* disrupt the macrophage actin filament network (Guerin & de Chastellier, 2000). Griffiths group showed that actin assembly by isolated latex beads phagosomes (LBP) correlates with their maturation status (Defacque et al., 2000). This *in vitro* assay monitors the polymerization of rhodamine-actin by phagosomal intact membranes in the presence of ATP and thymosin-β4 and in the absence of cytosolic extracts. The actin nucleation machinery, which includes the ezrin, radixin, moesin (ERM) protein family and phosphatidylinositol phosphates PIP and PIP2, are part of the membrane. Thymosin-β4 is used to prevent spontaneous actin nucleation at a concentration such that the phenomenon only occurs if a stimulator effector will induce the system. The standard assay works better at low ATP concentrations (0.2 mM) and the percentage of phagosomes that present red

actin dots in the LBP system is around 10-30% (Figure 1.A). At physiological concentrations of ATP (5mM) the system is blocked unless a stimulator will activate actin assembly (Defacque et al., 2000). Since LBP make cAMP at high ATP but not at low, via protein kinase A and cAMP is an inhibitor of actin assembly this may provide an explanation why the system is blocked at physiological concentrations of ATP (Kalamidas et al., 2006).

Intriguingly *in vitro*, phagosomes containing live pathogenic *Mycobacterium* failed to induce actin assembly, whereas phagosomes containing avirulent or dead mycobacteria nucleated actin readily depending on the age (as for the latex beads system)(Anes et al., 2003) (Figure 1.B).

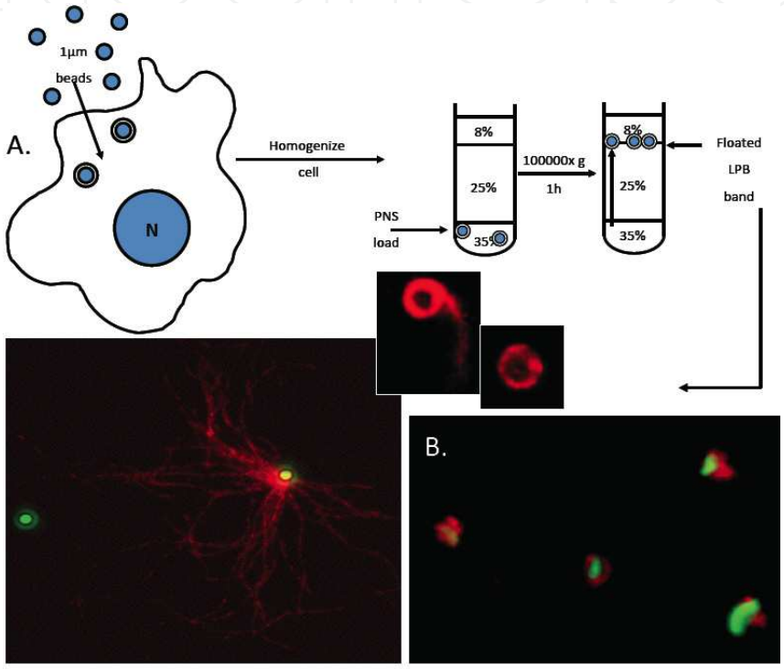

Fig. 1. Actin nucleation/assembly assay (red). (A) latex beads containing phagosomes. (B) *Mycobacterium smegmatis*-containing phagosomes

We have accumulated a large set of correlative observations such that all conditions we have identified as being stimulating in the in *vitro actin* assembly assay also stimulated actin accumulation around phagosomes *in vivo*, phagosome fusion with late endosomes and lysosomes and the killing of mycobacteria, a consequence of phagolysosome fusion (Anes et al., 2003, Anes et al., 2006) (Figure 2).

Conversely, compounds that inhibited *in vitro* actin assembly do not increase F-actin on phagosomes fail to promote phagolysosome fusion and favour growth of intracellular mycobacteria. Models have been developed to explain how actin filaments nucleated from the surface of a membrane organelle could "attract" other bound organelles toward it (Kjeken et al., 2004) (Figure3). Therefore we found conditions were phagosome maturation blockade by *M. tuberculosis* could be reversed to some extend by treating host infected cells with effectors that induced actin assembly and phagolysosome fusion with concomitant lumen acidification.

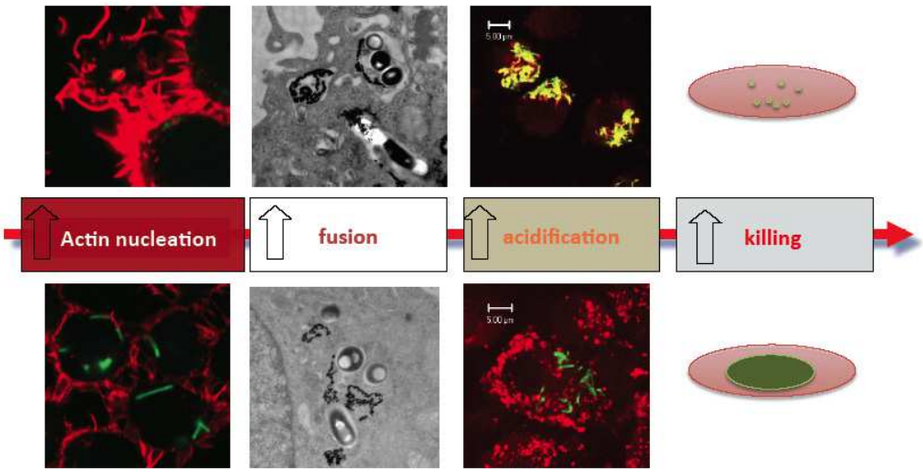

Fig. 2. Actin assembly *in vivo*: conditions that increase actin in phagosomal membranes lead to increase membrane fusion with lysosomes, acidification and mycobacteria killing. Left panel: confocal microscopy; (red) F-actin rhodamin-phaloidin , (green) Mtb-GFP. Middle panel: EM of gold-containing lysosomes and fusion with phagosomes. Right panel, acidotrophic lysotracker red vesicles and phagosomes containing heat killed Mtb (green).

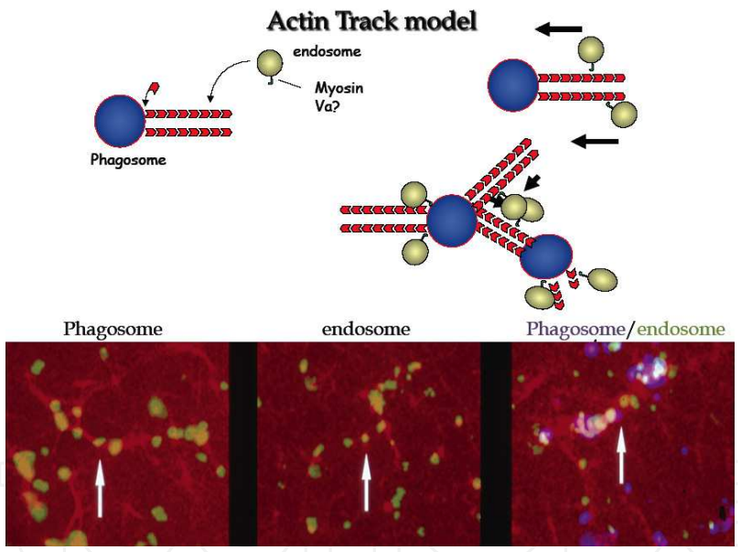

Fig. 3. The actin track model. Actin filaments nucleated from the surface of a membrane organelle could "attract" other bound organelles toward it helping fusion events. Confocal microscopy showing aggregation of phagosomes with endosomes along actin filaments (red).

4. Reversion of phagolysosome blockade by host effectors: Role of lipids, ATP, P2X7 receptor on actin assembly and on mycobacteria phagosome maturation

Phagosome maturation is known to be influenced by the lipid species present on its membrane, although studies published have focused mostly on the kinase that generates phosphatidyl i n o s i tol-4-phosphate (PIP), and on PtdIns(4,5)P2 (PIP2), which binds actin

nucleation proteins (Vieira et al., 2002). The key role of PIP and PIP2 opened the door for the analysis of all lipids that interconnected with these phosphoinositides in the actin assembly process, as well as sphingolipids and fatty acids (Anes et al., 2003).

The notion that *M. tuberculosis* may control phagosome maturation through the modulation of host lipids has been suggested by previous studies (Fratti et al., 2003), but the possible lipid mediators were not characterized. We showed that the enrichment of certain lipid, to mycobacteria-phagosome membranes stimulates the nucleation of actin and therefore phagosome maturation boosting the killing ability of macrophages to mycobacteria (Anes et al., 2003, Jordao et al., 2008). The F-Actin Stimulatory Factors (ASF) in the above experiments are the eicosanoide omega 6 arachadonic acid (AA), ceramide (Cer) and sphingosine-1-phosphate (S1P).

An important role of ATP here is to be used by kinases to produce PIP, PIP2, S1P, ceramide-1-phosphate (Cer1P) and phosphatidic acid (PA). All these phosphorylated lipids are stimulatory of F-actin at physiological concentrations of this purine. The incorporation of these phosphorylated lipids into the phagosomal membrane in the presence of ATP and ADP led to the translocation of ADP, but not ATP across the phagosomal membrane into the lumen. Once there, this ADP is converted to ATP by adenylate kinase activity. We propose that luminal ATP accumulates in response to selected lipids and activates the purinergic receptor P2X7R that signals across the phagosomal membrane to trigger actin assembly on the cytoplasmic membrane surface. (Kuehnel et al., 2009). P2X7R is a cationic channel, identified in the J774 macrophage latex bead phagosomes proteome (M. Desjardins, personal communication) that is able to bind via its cytoplasmic domain to a complex of 12 proteins, including actin. Whereas LBP prepared from wild-type P2X7R- positive bone marrow macrophages could assemble actin and was stimulated by S1P or PIP at physiological concentrations of ATP, the equivalent LBP from P2X7R-knock-out mice macrophages failed to be stimulated by these lipids. This argues for a model in which PIP and S1P induce ATP accumulation in the phagosome lumen. This ATP then activates the P2X7R that signals downstream of the phagosome actin assembly machinery (Kuehnel et al., 2009, Kuehnel et al., 2009b). From all these studies described above, its is now becoming clear that phagosomes are not passive vesicles that only acquire proteins and lipids via vesicular transport, or (for proteins) directly from the cytoplasm, and lose these components by recycling or dissociation. These organelles are also themselves capable of a plethora of biochemical activities, for example the enzymatic synthesis of different lipids. A dynamic description, including a predictive model of the interactions of lipids linked to PIP2 has recently been performed. This includes a systems level analysis of these lipids and other molecules on phagosomal membranes (Kuhnel et al., 2008).

The inhibitors (AIF: for F-Actin inhibitor factors) include the eicosanoides omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Anes et al., 2003) and phosphatidylcholine (PC) (Treede et al., 2007). This group of lipids enriched in the membranes of infected cultures of macrophages protected the bacteria inducing the intracellular growth of *M. tuberculosis* and other mycobacteria.

4.1 Eicosanoides, important immunoregulatory and host defence mechanisms in TB

The omega 3 and omega 6 ratio are important for immunoregulatory and host defence functions. A striking effect on phagosomal-actin assembly, late fusion and killing of

mycobacteria was seen when a number of pro-inflammatory lipids, especially the omega-6 fatty acid, arachidonic acid (AA), were added to J774 macrophages infected with the non-pathogenic *M. smegmatis* or with virulent strains of *M. tuberculosis*. In contrast, the anti-inflammatory omega-3 fatty acid eicosapentaenoic acid (EPA) induced an increase in mycobacterial growth in macrophages. These results were exciting because they fitted nicely into a general pattern seen with these groups of lipids in whole organisms. In support of this, three earlier studies showed that diets rich in omega-3 fatty acids led to significant increases in growth of *Salmonella* in mice (Chang et al., 1992) and *M. tuberculosis* in guinea pigs (Mayatepek et al., 1994, Paul et al., 1997).

Indeed during the last years we have followed the effects of these lipids and their molecular effectors on the outcome of the infection in more complex systems than the macrophage cell culture model. Collectively, our data suggests that a high omega-6 fatty acids diet might be beneficial against mycobacteriosis, while a high omega-3 fatty acids diet might be detrimental. However, depending on the molecular effectors and, on the time during infection that the lipid is supplemented, opposite signaling effects were observed. The data with Koch's bacilli raise an important public health question: are those individuals latently infected with *M. tuberculosis*, but asymptomatic at increased risk of reactivating the disease upon omega-3 fatty acids supplementation?

Given this increasing tendency to advise and use omega-3 dietary supplements, which have beneficial effects among uninfected individuals it is important to evaluate their effect on patients suffering from tuberculosis and other infections since their impact on disease evolution is unknown. A literature search on the effects of omega-3 and -6 lipids on a variety of different pathogens, especially in animal models, the conclusion suggest that polyunsaturated fatty acids are not generally beneficial and are often detrimental. In a critical evaluation of all the experiments done to test the effects of omega-3 lipids in the context of infectious diseases Anderson and Fritsche concluded that there were "an equal number of papers published that report an adverse effect of omega-3 fatty acids on host infectious disease resistance as those that do not show an effect or show a beneficial effect"(Anderson & Fritsche, 2002).

The results obtained by our group were surprising in that the opposite effects were seen in the animals, compared with observations in macrophages. For mycobacteria infected macrophages the omega-3 fatty acid, EPA, enhanced intracellular survival of *M.tuberculosis* while in infected mice an omega-3 fatty acid enriched diet promoted bacteria killing. When we looked at the effects of an omega-6 fatty acid enriched diet on the intracellular survival of intracellular pathogens we found no effect on *M.tuberculosis* infected mice while for salmonella infected animals the bacteria survival was improved (Jordao et al., 2008). From the perspective of actin assembly, our results were in agreement with the salmonella model because AA, by stimulating actin assembly will create an even higher actin meshwork around the salmonella containing vacuole preventing membranes contact and therefore fusion with lysosomes, thus protecting the bacteria. For the mycobacteria model the results, in infected macrophages, also agree with our model as AA, by increasing actin around *M.tuberculosis* phagosomes will help bacteria killing. In animals it may be the case that downstream breakdown products of the omega-6 fatty acid added to the diet could play opposite and more complex effects thus reflecting the lack of agreement regarding the outcome of the infection.

Eicosanoids are lipids responsible for many of the effects found in acute inflammation. Depending on the nature of the stimulus and the local pulmonary environment, the alveolar macrophage can be triggered to selectively release AA from membranes (Cochran et al., 1987). Free AA thus becomes the substrate for the ciclooxygenase (COX) and 5-lipoxygenases (5-LO) enzyme cascades that convert AA to a complex array of prostanoids, leukotrienes and lipoxins. The inflammatory response play a key role in shaping the adaptive response in large part through the secretion of an array of mediators such as interleukin 1β (IL-1β), IL-18, IL-12, tumor necrosis factor α (TNF-α), prostaglandin E2 (PGE2), leukotriene B4 (LTB4) and lipoxin A4 (LXA4) in response to the pathogen. EPA, another major source of eicosanoids present in membranes, inhibits these conversions and has a general anti-inflammatory role. However the enrichment of a membrane with EPA will transiently induce AA release that will become free for COX and 5-LO enzyme cascades. The long time enrichment of membranes with EPA will lead to AA depletion and to anti-inflammation by preventing AA conversion into the group of potent pro-inflammatory modulators. Furthermore it will generate a class of PGs of the 3-series that are far less pro-inflammatory than those of the 2-series (Calder, 2002).

Recently was found that the membrane of latex bead containing phagosome has the enzymes required to convert AA to a number of prostaglandins (PGs) (Griffiths G., personal communication). Moreover, some of these PGs, such as PGE2 inhibit actin assembly by LBP. In contrast, AA the precursor of PGE2, is a potent stimulator of phagosomal actin assembly. Here depending on whether there is accumulation of the precursor (AA) or the ending product (PGE2) opposite effects will be observed on the ability of phagosomal membranes to assemble actin. AA stimulating actin assembly will induce phagosome maturation and lysosomal pathogen killing while its product, with an inhibitory effect on actin assembly, will contribute to pathogen survival.

5. Control through the transcription factor NF-κB on phagosome maturation and macrophage activation by signaling lipids

A critical regulator of genes involved in inflammation is NF- κB (Caamano & Hunter, 2002). This transcription factor consists of two subfamilies: the 'NF-κB' proteins and the 'Rel' proteins which are present in the cytoplasm as hetero-dimers, in a complex with an inhibitor, IκB. When pro-inflammatory signaling occurs via activation of cell surface receptors such as the Toll-like receptors (TLR), IκB becomes phosphorylated. This releases the active subunits that enter the nucleus, where they up-regulate the transcription of hundreds of genes, a reflection of the complexity of this part of the inflammatory response (Natoli et al., 2005). In a recent study we showed that NF-κB is transiently activated early after infection of J774 cells and primary bone marrow derived macrophages with *M. smegmatis*. This activation is essential for mycobacterial killing since when NF-κB is blocked *M. smegmatis* survives. Using microarray analysis, we identified many lysosomal enzymes and membrane-trafficking regulators, including cathepsins, LAMP-2 and Rab34, were regulated by NF-κB during infection (Gutierrez et al., 2008). Our results argue that NF-κB activation increases the synthesis of membrane trafficking molecules, which may be rate limiting for regulating phagolysosome fusion during infection. The direct consequence of NF-κB inhibition is the impaired delivery of lysosomal enzymes to *M. smegmatis* phagosomes and reduced killing.

Whether or not pathogenic mycobacteria activate NF-κB is not clearly established, with different groups reporting conflicting results. Using confocal microscopy and ELISA, we observed a low and transient activation of NF-κB by *M. avium*. Similar results were reported before (Lee & Schorey, 2005). In contrast, others observed that high and low virulence strains of the *M. avium*-intracellular complex (MAC) activated NF-κB (Giri et al., 1998). There are similar reports of NF-κB activation in macrophages infected with *M. tuberculosis*. However, many studies argue that the pathogens strongly block the proinflammatory response in general (Beltan et al., 2000). Based on our observations, we suggest the model in which pathogenic mycobacteria induce NF-κB activation upon contact with the macrophage. As soon as the mycobacteria start the synthesis and secretion of virulence effectors, the system may be repressed. A hitherto unappreciated benefit to the pathogen in inhibiting the NF-κB system is the consequence that the phagolysosome fusion events would also be blocked. In summary, our results pinpoint a novel role of the NF-κB system to allow (directly or indirectly) the synthesis of molecules involved in intracellular trafficking. This regulation is linked to phagolysosome fusion, which facilitates killing of intracellular mycobacteria. In addition, our microarray screen identified a large number of potential new regulators of phagolysosome fusion; NF-κB is thus a key regulator of factors that facilitate the intracellular killing of mycobacteria.

Many links have been described between lipids and NF-κB activation in different systems. For example, in Caco-2cells AA activates NF-κB whereas EPA had no effect (Ramakers et al., 2007). Moreover, in the same cells phosphatidylcholine, which inhibits LBP actin assembly, inhibited NF-κB activation induced by TNF- α (Treede et al., 2007).

In macrophages, sphingomyelin (SM), PIP and AA enhanced NF-κB activation and the cell surface expression of CD69, a macrophage activation marker regulated by NF-κB. Sph, S1P, EPA and PC failed to activate either NF-κB or CD69. Cer activated CD69 expression without activating NF-κB. In *M. smegmatis*-infected macrophages, SM, PIP and AA transiently activated NF-κB in a manner that was enhanced. In contrast *Mycobacterium avium* mostly repressed NF-κB activation and only SM and AA could induce its partial activation. While lipids that activate NF-κB in uninfected cells tend to kill mycobacteria in macrophages Sph and S1P failed to activate NF-κB under most conditions but nevertheless enhanced killing of *M. smegmatis*, *M. avium* and *M. tuberculosis* H37Rv. Our results argue that both NF-κB-dependent and -independent mechanisms are involved in macrophage killing of mycobacteria and that both mechanisms can be enhanced by selected lipids (Gutierrez et al., 2009).

As stated above, NF-κB signaling could be also induced by TNF-α. TNF-α signaling involves binding to members of TNF receptor super-family and recruitment of a complex of adapter proteins. Among these, TNF-receptor associated factors (TRAFs) activate several intracellular signal transduction pathways, in particular NF-κB and, Map kinases (MAPKs) that lead to modulation of gene expression by different transcription factors. Pathogenic mycobacteria tends to inhibit all these pathways (Gutierrez et al., 2008, Schorey & Cooper, 2003).

MAPKs are central players in cell signaling and much of their activities are localized on membranes. There are three different classes of these kinases, namely ERK, JNK and p38. A

number of studies have shown that these kinases are activated upon infection with mycobacteria (Schorey & Cooper, 2003). Moreover, p38 and ERK are activated more during infection with non-pathogenic compared with pathogenic mycobacteria, implying that the pathogens inhibit these kinases (Roach & Schorey, 2002). The kinase p38 has been implicated in early endosome fusion; Fratti and colleagues also associated p38 activation with an inhibition of phagosome maturation in cells infected with *Mycobacterium bovis* (BCG) (Fratti et al., 2003). We additionally provided evidence for its importance in regulating phagosomal actin assembly (Anes et al., 2006). By inhibiting this kinase, a block on phagosome actin assembly *in vitro* was observed. *In vivo* p38 inhibition, blocked phagosome maturation and, increased survival of *M. smegmatis* within J774 macrophages. We also found that in *M.tuberculosis*-infected macrophages TNF-α secretion was stimulated by treatment with AA, whereas EPA inhibited this process (Jordao et al., 2008b). In line with this, AA strongly activated the pro-inflammatory MAPK p38 in uninfected cells but *M.tuberculosis* infected cells blocked the ability of AA to activate p38 leading us to conclude that AA-dependent killing is therefore independent of p38.

6. Control through programmed cell death and inflammation: Inflammasome activation by Koch's bacilli

Several intracellular pathogens, including Leishmania, Coxiella, Salmonella, Chlamydia and Yersinia, induce host-cell apoptosis as a way of minimizing the inflammatory response and avoid detection (Bergsbaken & Cookson, 2007, John & Hunter, 2008, Peters et al., 2008). In some of these cases, the induction of host-cell necrosis, sometimes by pyroptosis, leads to lower bacterial viability and is beneficial to the host (Haimovich & Venkatesan, 2006). Other acute bacterial pathogens, such as Pseudomonas, Neisseria and Streptococcus, are cleared by host-cell apoptosis, and these pathogens evade innate immunity by inhibiting apoptosis (Tunbridge et al., 2006). Virulent strains of *M. tuberculosis* induce necrosis of both human and mouse macrophages, whereas attenuated *M. tuberculosis* strains or other nonpathogenic mycobacterial species generally do not (Divangahi et al., 2009). In addition, there is accumulating evidence that apoptosis, whether induced by the pathogen itself, pharmacologically or by cytotoxic lymphocytes results in lower viability of *M. tuberculosis* (Duan et al., 2001, Gan et al., 2008, Oddo et al., 1998).

One strategy used by *M. tuberculosis* to avoid apoptosis is the subversion of host eicosanoid biosynthetic pathways. The finding that *M. tuberculosis* infection significantly increases the membrane release of AA via activation of cPLA2 γ (Duan et al., 2001) and that mice knock-out for 5-LO are more resistant to *M. tuberculosis* infection (Bafica et al., 2005) raised the question of whether eicosanoid production is involved in macrophage necrotic programme cell death and inhibition of apoptosis induced by virulent *M. tuberculosis*. The attenuated *M. tuberculosis* strain H37Ra induces the production of prostaglandin E2 (PGE2), which protects the mitochondrial inner membrane and induces the repair of plasma membrane microdisruptions inflicted by the pathogen (Keane et al., 2000). These events protect the host macrophages against necrosis and instead promote apoptosis. In contrast, intra-cellular infection with the virulent H37Rv strain of *M. tuberculosis* induces the production of lipoxin A4 (LXA4), which inhibits cyclooxygenase production and PGE2 biosynthesis. In a PGE2-poor microenvironment, macrophages cannot prevent the mitochondrial damage or repair the plasma membrane disruptions caused by *M. tuberculosis*, and this leads to

necrosis. Virulent *M. tuberculosis* in pre-necrotic macrophages continues to replicate and to spread to uninfected macrophages after the cells are lysed. Thus, the balance of PGE2 and LXA4 regulates the relative amounts of apoptosis and necrosis after *M. tuberculosis* infection, with important functional consequences for innate control of the intracellular infection.

Pyroptosis in the context of tuberculosis is controversial: from one side it leads to potent pro-inflammation that drives the active disease; from other side allows the release of intracellular bacteria and their escape to hydrolytic digestion. At one point in the life of an intracellular Koch's bacillus, there comes the time to exit the cell and infect the next one. In the larger scale of the human infection, mycobacteria need to access a novel host organism. In the infectious stage bacilli are coughed up and aerosolized within microdroplets. Dissemination of the infection therefore requires leaking of bacilli out of caseating necrotic foci or caverns into the alveolar space, which is promoted by exacerbated inflammation and massive cell death. Intranasal lipopolysaccharide treatment simultaneously to *M. tuberculosis* infection in mice induced necrotic lesions, otherwise not seen in this animal model for TB, indicating the importance of inflammatory stimuli for caseating necrosis.

This necrotic programme of cell death results from the assembly of inflammasomes, a complex of pattern recognition cytosolic receptors (NLRs: NOD-like receptors) (reviewed by (Sirard et al., 2007)) and adaptor proteins containing caspase recruitment domains (CARD) which are able to recruit and activate pro-caspase-1. Mature Casp-1 then activates the isoforms of pro-IL-1β and pro-IL-18 and the active pro-inflammatory interleukins are then secreted through exosomes, concomitant or not with induction of pyroptosis. In humans, the NLR family has at least 20 members. Structuraly, NLRs are characterized by their nucleotide-binding oligomerization domain (NOD), a leucin-rich domain that recognizes pathogen associated molecular patterns (PAMPs) and a signalling domain that either triggers NF- κB, or other signaling platforms such as MAPK, type 1 interferon pathways or induces inflammasome activation.

Different NLRs associate with each other and CARD-containing protein adaptors to form diverse inflammasome complexes in response to different bacterial elicitor molecules or endogenous danger signals. For example, the *Salmonella* and *Legionella* flagellin proteins are sensed by an NLRC4/CARD12/CLAN inflammasome (Mariathasan et al., 2004) whereas muramyl dipeptide (MDP) is sensed by NLRP1 and NLRP3 inflammasomes (Martinon et al., 2004). Various microbial toxins have also been shown to activate the inflammasome (Nour et al., 2009). The NLRP3/CIAS1/Cryopyrin inflammasome is activated either by uric acid crystals formed during gout, silica, asbestos or cathepsin B as well extracellular ATP released from necrotic cells (Cassel et al., 2008, Mariathasan et al., 2006).

M. tuberculosis resides largely within a phagosome-like compartment of host macrophages during infection (Jordao et al., 2008). Inflammasome activation it is postulated to occur only if these effectors or products from a signalling cascade reach the cytosol and gain access to NLRs. Despite its apparent sequestration from the cytosol, *M. tuberculosis* has been shown to trigger NLR pathways that induce type I IFN expression. This recognition event depends on the ESX-1 specialized protein secretion system of the bacterium (Pandey et al., 2009). ESX-1 has been shown to be involved in the perturbation of host cell membranes, and has therefore been hypothesized to promote type I IFN production by facilitating the delivery of bacterial products into the cytosol including those responsible for secretion of the virulence-

associated antigens ESAT-6 and the CFP-10 (Stanley et al., 2007). Indeed this is in agreement with recent findings that support the escape of *M. tuberculosis* from the phagosome to the cytosol at latter times post-infection in human primary macrophages and DCs (van der Wel et al., 2007). Some groups claimed that *M. tuberculosis* blocks inflammasome activation (Master et al., 2008). However part of the experiments were performed using BCG and because this attenuated strain had lost the complete RD1 region that codifies for ESX-1 and ESAT6 is unable to secrete important PAMPs to the cytosol that may be envolved in inflammasome activation. The same logic is applied for the use of the attenuated *M. tuberculosis* H37Ra, which has a mutation in PhoP which inhibits ESX-1 function, predominantly prevents necrosis and leads to sequestration and decimation of the intracellular bacteria (Chen et al., 2006). It has previously been shown that the secreted mycobacterial protein ESAT-6 in its purified form induces cell death in macrophages (Derrick & Morris, 2007) and that the protein has membrane-lysing activity (Smith et al., 2008). This makes ESAT-6 an attractive candidate as the bacterial factor responsible for necrosis. Lerm group found that deletion of the gene encoding ESAT-6 or of the RD1 region led to abrogation of the necrosis-inducing effect of *M. tuberculosis* as well as diminished IL-1β secretion by the host macrophage (Welin et al., 2011).

Recent studies have demonstrated that casp-1 activation and subsequent IL-1β synthesis are strongly upregulated in *M. tuberculosis*-infected macrophages (Montero et al., 2004), and inflammasome activation occurs upon *M. tuberculosis* infection (Netea et al., 2006). Finally, *M. tuberculosis* infection was proved to be sensed by NOD2, a cytosolic NLR that recognizes a component of peptidoglycan found in mycobacterial cell wall (Ferwerda et al., 2005). Another mycobacterial protein, a metalloprotease was so far the unique demonstrated to suppress the IPAF/NLRC4 inflammasome (Master et al., 2008).

Our group hypothesized that inflammasome activation is triggered by *M. tuberculosis* components that cross the phagosomal barrier where they are sensed by cytosolic pattern recognition receptors (PRRs) to activate the inflammasome and thereby promote IL-1β maturation. It was demonstrated that casp-1 activation depends on live intracellular bacteria that express a functional ESX-1 system. The contribution of ESX-1 appears to be attributable to the ESAT-6 protein, which facilitates the diffusion of other bacterial products such as AG85 into the cytosol. Casp-1 activation was found to be increased after co-challenge of individual *M. tuberculosis* PAMPs such as AG85, PIM1/2, mAGP, Man-LAM and PGN (Mishra et al., 2010).

To identify the components of the inflammasome most important during *M. tuberculosis* infection, a shRNA-based screen was carried out to silence individual NLR and CARD proteins selectively in THP-1 monocytes. These cells were transduced with a panel of lentivirus-encoding shRNAs that targeted 22 NLRs and 14 CARD domain-containing proteins in the human genome. We further identify an NLRP3 and ASC-containing complex as the major inflammasome activated in macrophages by *M. tuberculosis* infection, and implicate a number of other NLR and CARD proteins in this process. Nine genes were considered to be either positive or negative regulators of inflammasome function during *M. tuberculosis* infection, six genes played a role upon ESAT-6 stimulation, and 14 genes were important upon dual stimulation with ESAT-6 and AG85. Most of the shRNAs that inhibited IL-1β secretion in ESAT-6 pulsed cells also inhibited this response when ESAT-6 was added together with AG85. Most importantly, knock-down of NLRP3/CIAS1, PYCARD/ASC and

CARD6 resulted in a similar inhibition of IL-1β secretion in all the three conditions tested (Mishra et al., 2010).

In addition to NLRP3 and ASC, we found that depletion of CARD6 also impaired IL-1β secretion in response to all stimuli. CARD6 has been shown to positively regulate NF-kB activation (Dufner & Mak, 2006). Although CARD6 was important for IL-1β secretion in response to both live bacteria and isolated components, we were not able to detect a physical interaction between CARD6 and the other inflammasome components including casp-1. Thus, we speculate that CARD6 may not be involved directly in inflammasome assembly during *M. tuberculosis* infection, but may promote IL-1β production by regulating the synthesis of proIL-1β by activating NF-kB family of transcription factors. Consistent with the previously published reports, we identified NOD2 as one of the sensors for *M. tuberculosis* infection.

IL-1β secretion was also impaired upon depletion of CARD12/NLRC4 when cells were challenged with ESAT-6 plus AG85, which might suggest the involvement of more than one inflammasome platform in the regulation of IL-1β release during *M. tuberculosis* infection. The mycobacterial cord factor Trehalose-6, 6-dimycolate (TDM; also called cord factor) is a mycobacterial cell wall glycolipid that may be the most studied immunostimulatory component of *M. tuberculosis*. Macrophages detect TDM through a recently identified C-type lectin receptor-'Mincle' and produce inflammatory cytokines (Ishikawa et al., 2009). This activation most likely proceeds through a Syk-CARD9-Bcl10-Malt1 signalling pathway to induce a specific innate activation programme that is distinct from the response to TLR ligands (Werninghaus et al., 2009).

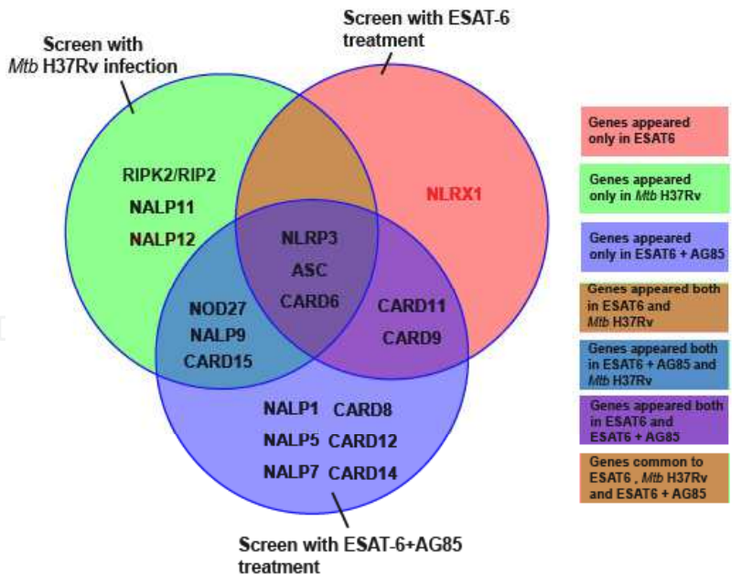

Fig. 4. NLR's and CARD-containing protein adaptors that responds to Koch's infection and to major PAMPs ESAT-6 and ESAT-6 co-challenged with AG85.

Recent reports have identified CARD9 as a central integrator of various PRR signaling pathways (Colonna, 2007). We observed that a component of the IL-1β secretion in macrophages challenged with ESAT-6 and with ESAT-6 plus AG85 depended on CARD9.

Together these data support an important role for CARD9 in the innate immune response triggered by *M. tuberculosis* infection, ESAT-6, AG85 and TDM. In conclusion our data suggest a previously unrecognized role for ESAT-6 in aiding the access of mycobacterial PAMPs such as AG85 to the macrophage cytosol eliciting casp-1 activation and IL-1β secretion. The observation that IL-1β and NLRP3 mRNA is induced in macrophages derived from patients with active TB indicates that a similar inflammatory pathway is likely to be important for the pathogenesis of human TB (Mishra et al., 2010).

Targeting cytosolic receptors or their adapters and therefore blocking the most relevant inflammasomes to be assembled during latent infection may prevent the active disease by decreasing pro-inflammatory responses that drives intracellular bacteria release from macrophages and the necrosis of the granuloma.

7. Conclusion

From the above review of the literature, it is evident that the interaction between the Koch's bacillus and the host macrophage is essential in determining the outcome of infection. However, the way in which the bacteria subvert the response of the macrophage originally designed to kill is still not completely understood. There are gaps in the knowledge, including how the bacteria inhibit phagosomal maturation and how the bacilli induce cell death. Furthermore, although a lot of effort has gone into studying phagosomal maturation in the context of *M. tuberculosis*, it is still not clear what determines the outcome of infection. The deciding factors for overcoming the block in phagosomal maturation, the crucial determinants in controlling the bacilli and disabling their replication, and the activation factors necessary to enhance macrophage function to remove the infection are not fully known. The dogma that inhibition of phagolysosomal fusion is the determinant factor that allows mycobacterial growth has not been well substantiated in a human system. Furthermore, methods designed to study these factors are needed to enable a better understanding. Many pieces of information come from studies of mouse or other animal models, and are based on results obtained with avirulent species of mycobacteria and the extrapolation of these to the human *in vivo* situation is often difficult.

The most pressing questions that emerge from the current body of data on *M. tuberculosis* fall into two discrete categories. First, how does the bacterium modulate the endocytic network of its host cell? Although the answer to this question is important for our understanding of the bacterium's success as a pathogen, it is unlikely to give rise to a treatment for infection because reversal of this phenotype would probably be difficult to achieve locally, and systemic modulation of endosome–lysosome fusion does not strike me as a feasible solution. Second, how can our appreciation of the environment in which the bacterium finds itself be exploited to result in new or improved ways to fight infection? Understanding the position of the vacuole within the endosomal continuum could lead to improved drug delivery. Most important, however, is the need to increase our knowledge of the intralumenal environment within the vacuole. If we want to screen for, and isolate, new drugs against relevant targets, we have to develop a more detailed understanding of the environment within the lumen of the endosomal vesicles. A huge amount of activity has been devoted to membrane fusion and its modulation by cytosolic mediators. A much greater understanding of the content of the endosomal–lysosomal milieu is needed. What are the concentrations of metal ions? What is the form and abundance of lipids and fatty

acids? These questions are key to our appreciation of both intracellular infection and to our understanding of the physiology of the endosomal system.

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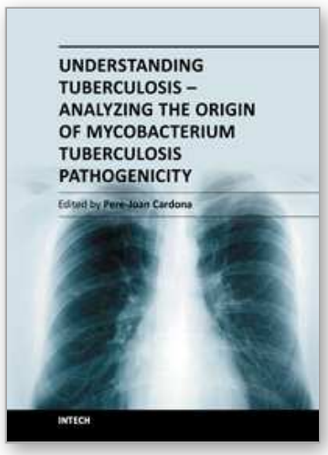

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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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