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**Apoptotic neutrophils enhance the immune response  
against *Mycobacterium tuberculosis***

**Y. Alexander Z. Persson**



**Linköping University**  
**FACULTY OF HEALTH SCIENCES**

Division of Medical Microbiology  
Department of Clinical and Experimental Medicine  
Faculty of Health Sciences  
Linköping University  
SE-58185 Linköping, Sweden

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This is my book

-And I owe it all to my family



*The great tragedy of science*  
*-The slaying of a beautiful hypothesis by an ugly fact*

**Thomas Henry Huxley** (Darwin's Bulldog)  
British biologist  
\*1825 †1895



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## Abstract

*Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis, a disease that for years was considered to belong of the past, but tuberculosis is back causing over 2 million deaths per year. The infection can be dormant for decades and an active immune response can prevent the infection from progressing into active disease. However, the HIV/AIDS epidemic has caused an alarming rise in tuberculosis cases.

The main infectious route for Mtb is through the airways into the lungs, where they encounter alveolar macrophages. Mtb are phagocytosed by these macrophages, but instead of being killing within the phagosome, Mtb modulates the cell to become a host in which the bacteria thrive. The lack of capacity to eradicate the infection stimulate cells of the immune system to gather around infected macrophages and form a granuloma that walls off the infection. Within this granuloma, Mtb can wait silently and later progress into active disease. However, only a fraction of exposed individuals develop disease, indicating that initial eradication of Mtb infections is possible. Such immediate response must be directed by the innate immunity comprised of phagocytes such as neutrophils (PMNs) and non-activated macrophages. Upon Mtb infection, macrophages become anergic and PMNs enter apoptosis. PMNs have a short lifespan and are cleared by neighbouring phagocytes, a mechanism described to resolve the inflammation and modulate tissue regeneration.

We found that Mtb-induced apoptosis in PMNs was not dependent on phagocytosis of the bacteria, indicating that Mtb have the capacity to induce apoptosis in multiple PMNs. Complement-mediated phagocytosis induce survival signals such as Akt in PMNs, but despite this, complement-opsonized Mtb was able to override the anti-apoptotic activation in the cells. Since phagocytes clear apoptotic cells, we investigated how clearance of Mtb-induced apoptotic PMNs affected macrophages. We found that Mtb-induced apoptotic PMNs inflicted pro-inflammatory activation of the macrophages that cleared them. In addition, this activation was mediated by Hsp72 released from the Mtb-induced apoptotic PMNs. Furthermore, apoptotic PMNs can work in synergy with phagocytosed Mtb to activate macrophages and enhance intracellular killing of Mtb.

Since dendritic cells are important for the regulation of immunity, we investigated whether Mtb-induced apoptotic PMNs affected the inflammatory response and maturation of dendritic cells. We found that Mtb-induced apoptotic PMNs trigger dendritic cells to enter a mature state able to activate naïve T-cell proliferation.

We propose that infected apoptotic PMNs is a potent activator of the inflammatory response during infections. Taken together, PMNs not only kill their share of pathogens but also modulate other immune cells, thereby forming a link between the early innate and the adaptive immune response during microbial challenge with Mtb.

## Svensk sammanfattning

Tuberkulos har länge varit en sjukdom som varit förpassad till historieböckerna. På grund av detta har forskningen stått stilla och utvecklingen av nya mediciner och vacciner stagnerat. Idag dör över 2 miljoner människor årligen och 1995 skördade tuberkulos fler liv än någonsin tidigare. Denna utveckling beror på flera faktorer såsom urbanisering och fattigdom, men framförallt på utvecklingen av HIV/AIDS, då HIV slår ut de immunceller som är ansvariga för att kontrollera bakteriens tillväxt i kroppen.

Tuberkulos orsakas av *Mycobacterium tuberculosis* (Mtb), en bakterie som sprids via luftvägarna. I lungan består kroppens försvar främst av makrofager, immunceller som äter upp och dödar bakterier. Trots att Mtb tas upp av dessa makrofager, klarar inte cellen av att döda bakterien. Detta gör att makrofagen blir en reservoar för bakterien som där ligger skyddad från övriga immunförsvaret. För att förhindra att bakterien sprids, omges dessa infekterade makrofager av en rad immunceller som bildar en barriär mellan bakterien och omgivningen. En sådan cellformation kallas granulom där bakterien kan ligga i decennier och vänta på rätt tillfälle att aktiveras, orsaka aktiv tuberkulos och spridas vidare.

Man vet dock att bara 10% av personer som blir exponerade för Mtb utvecklar sjukdom. Detta tyder på att de flesta individer har ett snabbt och effektivt immunsvaret som är kapabelt att döda bakterien innan granulom bildas. Ett sådant svar kan endast orsakas av det medfödda immunförsvaret som utgörs av neutrofiler och makrofager. I kontakt med Mtb dör neutrofilerna genom kontrollerad celledöd (apoptos). Neutrofiler har ett naturligt kort liv och lever i regel bara ett par dagar och apoptotiska celler städas bort av tex makrofager genom att de apoptotiska cellerna äts upp (fagocyteras). Detta fenomen är viktigt för att en inflammation ska upplösas och för att förhindra eventuell vävnadsskada.

Vår forskning visar att vid kontakt med bakterier försöker neutrofilerna skydda sig från att gå i apoptos men bakteriens förmåga att inducera apoptos är så kraftig att cellens överlevnadsförsök är överksamma. Eftersom makrofager fagocyterar apoptotiska neutrofiler, undersökte vi hur dessa Mtb-infekterade apoptotiska neutrofiler påverkar makrofager. Vi fann att apoptotiska neutrofiler kan modulera makrofagers förmåga att ta hand tuberkel bakterier och därmed stärka immunsvaret mot bakterien. De aktiverade makrofagerna inte bara frisläppte inflammatoriska mediatorer, utan Mtb-infekterade makrofager ökade sin förmåga att döda bakterien vid stimulering med apoptotiska neutrofiler. Detta berodde både på att apoptotiska neutrofiler direkt aktiverade makrofager, men även att de frisläppte

inflammatoriska mediatorer som verkade aktiverande på makrofager. Vidare fann vi att en annan inflammatorisk cell, den dendritiska cellen, som är viktig för uppkomsten av ett långvarigt immunologiskt minne, kraftigt aktiverades av Mtb-infekterade apoptotiska celler.

Vi visar att apoptotiska neutrofiler är en potent aktuator av inflammatoriska mekanismer vid infektioner. Förståelsen för hur celler interagerar med varandra och vad som styr ett lyckat immunsvaret mot Mtb-infektioner är betydelsefullt för utveckling av nya effektivare läkemedel och vaccinationer. Ett snabbt och effektivt immunförsvar är nödvändigt för att hindra spridning av denna allvarliga och mycket vanliga infektion.

## List of publications

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I           **A. Persson**, R. Blomgran-Julinder, D. Eklund, C. Lundström and O. Stendahl. Induction of apoptosis in human neutrophils by *Mycobacterium tuberculosis* is dependent on mature bacterial lipoproteins. Accepted for publication in Microb Pathog 2009.

II           **A. Persson**, R. Blomgran-Julinder, S. Rahman, L. Zheng, and O. Stendahl. *Mycobacterium tuberculosis*-induced apoptotic neutrophils trigger a pro-inflammatory response in macrophages through release of heat shock protein 72, acting in synergy with the bacteria. Microbes Infect 2008; 10: 233-40.

III           S. Hedlund, **A. Persson**, A. Vujic. O. Stendahl and M. Larsson. Dendritic cell activation by sensing *Mycobacterium tuberculosis*-induced apoptotic neutrophils via DC-SIGN. Submitted manuscript 2009.

IV           **A. Persson**, K. Svensson, D. Eklund and O. Stendahl. Apoptotic neutrophils activates the inflammatory response in macrophages –increased capacity to handle intracellular infection. Manuscript 2009.

## Abbreviations

BCG	Bacillus Calmette Guerin
CytC	Cytochrome C
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
iDC	Immature dendritic cell
MAPK	Mitogen-activated protein kinase
MPO	Myeloperoxidase
Mtb	<i>Mycobacterium tuberculosis</i>
NLR	NOD-like receptor
NO	Nitric oxide
NOS2	Nitric oxide synthase
PAMP	Pathogen associated molecular pattern
PMN	Neutrophil
PS	Phosphatidylserine
PSR	Phosphatidylserine receptor
RNI	Reactive nitrogen intermediate
ROS	Reactive oxygen species
Tb	Tuberculosis
TCR	T-cell receptor
TLR	Toll-like receptor





## Tuberculosis

*Mycobacterium tuberculosis* (Mtb) is one of the oldest known man killers and was even found during excavations of ancient gravesites (Daniel and Daniel 1999). Tuberculosis (Tb) affects mainly the lungs but can also disseminate to the central nervous system, joints, bones, blood or skin. In the late 80s Tb was uncommon in the industrialized parts of the world, but due to HIV/AIDS migration and inefficient treatment and surveillance, the number of new cases of tuberculosis has increased. However, in many parts of the world, tuberculosis was never suppressed and there are drug-resistant strains emerging at an alarming rate. WHO estimates that approximately one third of the worlds population is infected with Mtb and in the year of 1995 Tb killed more people than any other year in history (Dye, Scheele et al. 1999).

In Sweden today, there are approximately 400-500 cases annually but in the early 1900s, tuberculosis was one of the most common causes of death in Sweden and the only cure or at least improvement from the disease available at that time was a lengthy stay at a sanatorium. The first sanatorium was opened by the German physician Hermann Brehmer in the 1850s in the town of Görbersdorf, Germany. Dr Brehmer had discovered the health benefits of this type of treatment when he himself was cured during a trip to the Himalayas where he spent time at a sanatorium-like facility. The obvious health effects encouraged sanatoriums to be built all over Europe and in 1891 the first Swedish sanatorium opened in Mörsil. The legend has it that the Swedish king Oskar II wanted to build a modern iron warship, but queen Sofia managed to convince him to forget about the ship and to fight his war on Tb instead. Hence, Oskar II jubilee foundation installed three sanatoria to which people with Tb could come and rest, eat healthy foods, breathe fresh air and be frequently exposed to sunlight. Due to the fact that the pathogen is efficiently spread in crowded, dark environments with poor ventilation, the sanatoria were effective since they provided an environment that was anything but that. The effect of sunlight has since been proven beneficial not only through the direct effect on the bacteria but exposure to UV-light stimulates vitamin-D production. Activated vitamin-D in turn cleaves cathelicidin into the active form thus providing the infected cell with a potent anti-microbial peptide (Liu, Stenger et al. 2007).

Since the bovine pathogen *Mycobacterium bovis* is closely related to Mtb, it was long believed that the domestication of animals resulted in alterations of the bovine strains to adapt to human. In fact, genetic studies where the distribution and number of DNA deletions were compared (Brosch, Gordon et al. 2002; Mostowy, Cousins et al. 2002) have shown that Mtb was originally a human pathogen that transferred to cattle resulting in the development of *M. bovis*, a pathogen that today result in great monetary loss for the cattle industry. *M. bovis* can also infect humans, but following multiple passages in culture, the bacteria became attenuated. This new strain called BCG (bacillus Calmette Guerin) was 1921 used in human vaccination for the first time. In fact, BCG is still today the only vaccination available for Tb. Unfortunately the vaccine is mainly effective in small children to prevent the severe extrapulmonary forms of Tb and the vaccination of adults is shown to be less efficient (Fine 1995). A new effective vaccine to boost BGC immunity is therefore needed and great efforts are made worldwide to achieve this.

## **Mtb infection**

Mtb enters the host through inhalation into the alveolar space, where the bacteria encounter and become phagocytosed by alveolar macrophages, neutrophils (PMNs), and dendritic cells (DCs). The interaction between immune cells and Mtb initiates immunological responses essential for the recruitment of specific inflammatory cells as well as the development of granulomatous tissues (Orme and Cooper 1999; Davis and Ramakrishnan 2009), events described to be involved in both anti-mycobacterial immunity and Mtb pathogenesis (Kaufmann 2001; Tailleux, Schwartz et al. 2003; Bhatt, Hickman et al. 2004). Epidemiological studies pose evidence for several potential outcomes following exposure to Mtb via aerosol including immediate clearance of the bacteria, development of primary Tb or asymptomatic latent Tb that has the potential to transit into active disease (Dye, Scheele et al. 1999). The early eradication of Mtb is performed by innate immune cells before any adaptive immune response is activated and exposure to Mtb does in most non-vaccinated individuals not lead to infection as the bacteria are cleared before an infection is manifested or are harboured in the macrophage.

Despite being the most prevalent infectious disease claiming almost 2 million lives annually, only a minority of exposed individuals becomes infected and. It is today not known which immune responses are accountable for success or failure to clear the infection. Reactivation of an Mtb infection was convincingly demonstrated in a study where reactivation following 33 years of latent Tb was investigated by combining epidemiological evidence with molecular fingerprinting data (Lillebaek, Dirksen et al. 2002). An Mtb infection can thus be latent for years but does not necessarily lead to active disease as a healthy alert immune system can confine or may even sterilize the initial infection. In immune-compromised people (e.g. due to HIV-infection) the numbers of active Tb cases increase dramatically. Taken together, Mtb has an exceptionally potent ability to wait silently within the body for the right time to expand, a trait indicating that the best chance for the host (i.e. human) to survive is to efficiently eradicate the infection immediately. An efficiently and sufficiently activated innate immune response may play an important role in this process.

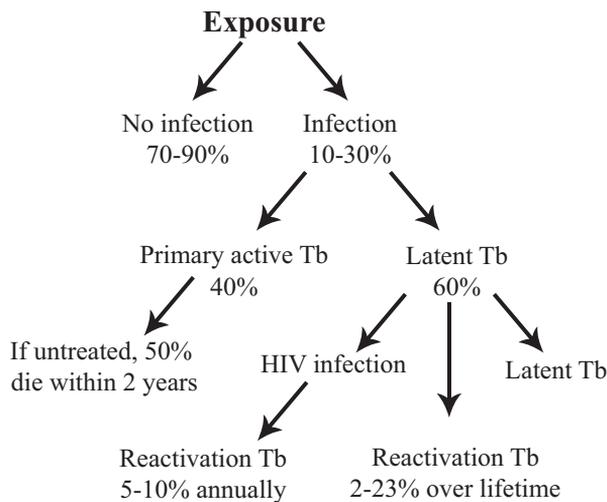


Figure showing the potential outcomes of exposure to Mtb.

## **Host defense against invading pathogens**

The human body has sophisticated ways of defending itself from invading pathogens. The immune system is comprised of specialized cells of the innate immunity that ingest and kill microorganisms. Some of these cells are capable of activating other cells of the immune system to form a massive inflammatory response and long-lasting immunological memory called adaptive immunity. For a long time the PMN was considered a quite simple cell whose main objective was to phagocytose and efficiently kill microorganisms. During the last decade, the view on this cell type has changed and the PMNs are constantly attributed new functions such as recruitment and modulation of adjacent cells.

Macrophages and DCs are professional phagocytes with capacity to ingest large preys in abundance. In addition to ingestion and killing of pathogens, these cells attract and present peptides derived from the ingested prey to induce an immune response specifically directed to the specific pathogen-derived peptides. Macrophage and DC activation is thus a prerequisite for the initiation of adaptive immunity.

The border between the two distinct branches of inflammation and host defense, innate and adaptive, is today not clear and a multitude of reports describes different immunological cells to display functions classically belonging to both of the distinct branches. Despite this, the immunological response will henceforth be described in terms of innate and adaptive, in lack of better description. The immediate on site response is maintained by cells of the innate immunity. To effectively meet the posing threat of an infection, this immune response has to be immediate, sufficient and coordinated. The main function of innate immunity is to rapidly clear microbes by phagocytosis and killing of the ingested prey before bacterial growth escalate. Following ingestion, macrophages and DCs present antigens derived from the ingested prey to cells of the adaptive immunity to build a long-lasting response and immunological memory. Tissue macrophages and DCs present at the site of infection initiates the initial inflammatory response by recruiting and activating additional inflammatory cells such as monocytes (subsequently differentiating into tissue macrophages) PMNs and lymphocyte subsets.

By migration to draining lymph nodes, DCs encounter an array of lymphocytes increasing the possibility of finding a lymphocyte that is specific for the antigen being presented. It is crucial that cells of the innate immunity are sufficient to control the initial infection since it takes the adaptive T-cell response, B-cell activation and specific antibody production up to two weeks to mobilize and reach sufficient clonal expansion.

A cell-independent way of killing pathogens is activation of the complement system which is composed of a variety of components present in serum. Engagement of the complement cascade destroys the bacterial wall, generates chemotactic mediators as well as leading to opsonization of the affected bacterium. The most predominant opsonin is C3bi which facilitate phagocytic uptake through scavenger receptors as well as specific CR3 receptors (Wright and Silverstein 1983). Opsonization of bacteria, by either complement factors or antibodies, does in most instances lead to death of the bacteria since the subsequent phagocytosis efficiently inactivate the bacteria. Following the onset of an adaptive immune response with antibody secretion, Fc receptors are the most predominant during phagocytosis of antibody-opsonized pathogens with actin-driven protrusions and rapid engulfment of the particle.

### **The macrophage – the gourmand**

Upon activation by tissue damage or infection, monocytes from the circulation migrate into the tissue. During this process, the cell gain qualities such as adherence to and migration through epithelial layers and tissue, increased phagocytic receptor expression and morphological characteristics for macrophages. Tissue macrophages are the main regulators of the inflammatory milieu during the initial phase of infection. This regulation is mediated by the vast repertoire of produced and released pro-inflammatory cytokines such as IL-1 $\beta$ , IL-12, IL-15 and TNF- $\alpha$ .

Macrophages are activated through stimulation with cytokines and by interaction with pathogens they ingest and kill. Following internalization, macrophages (however not as efficiently as PMNs) utilize NADPH oxidase to produce reactive oxygen species (ROS) in the

phagocytic vacuole. Nitric oxide synthase (NOS2) produced NO and subsequent reactive nitrogen intermediates (RNIs) are described to be a major effector in macrophage killing of phagocytosed microbes. Additional proton pumps contribute to acidification of the compartment and facilitation of the right environment for recruited hydrolytic enzymes such as cathepsin, all contributing to the killing and degradation of the internalized microbe (Beron, Alvarez-Dominguez et al. 1995; Desjardins 1995; Tjelle, Lovdal et al. 2000). Despite not having preformed granules, macrophages utilize antimicrobial peptides such as cathelicidin to inactivate the ingested prey. Cathelicidin is expressed upon receptor activation in a vitamin-D dependent manner (Liu, Stenger et al. 2006). Macrophages have also been shown to utilize antimicrobial component derived from granules originating from cytotoxic T-cells (Stenger, Hanson et al. 1998; Ochoa, Stenger et al. 2001) and PMNs (Tan, Meinken et al. 2006).

### **The dendritic cell – fine tuner of inflammatory response**

The DC is a professional phagocyte taking part both in the innate and adaptive immunity and are required for the initiation and maintenance of cellular immune responses against pathogens. Immature DCs (iDCs), located in peripheral tissues, constitutively sample the tissue microenvironment. Upon TLR signaling and capture of pathogens and processing of antigens, the cell enters a mature state. These mature DCs migrate to lymphoid organs where they initiate an adaptive immune response by presenting the captured antigens on MHC-II. CD4<sup>+</sup> T-cells, binds to the antigen/MHC complex by its TCR/CD3 and the following phosphorylation cascade leads to cellular activation (Constantin, Majeed et al. 2000). This interaction site between the antigen-presenting cell and the effector cell, called immunological synapse, is further stabilized by interactions between LFA-1 on the T-cell and ICAM on the DC. To engage in full activation, the CD4<sup>+</sup> T cells must acquire additional signals verifying that they are truly responding to a foreign antigen. These signals further tighten the immunological synapse and consist of interaction between T-cell CD28 and DC CD80 and CD86 resulting in activation of the CD28 and subsequent cellular activation and clonal expansion of activated CD4<sup>+</sup> T cells (Banchereau, Briere et al. 2000). Following this onset of adaptive immune expansion, the activated CD4<sup>+</sup> T-cells stimulate B-cell proliferation and production of antigen specific

antibodies as well as release of inflammatory cytokines such as IFN- $\gamma$  giving a specific immunity directed towards the infectious agent.

Also macrophages, in addition to phagocytosis and killing of microbes, have the capacity to present antigens and induce an adaptive immune response. However, the adaptive immune response takes about 10-14 days to reach full immunological potential. The very early immunological defense to invading pathogens therefore relies on phagocytic cells mainly of the innate immune system.

### **The neutrophil – the foot soldier of innate immunity**

Following phagocytosis, the ingested prey within the phagosome is subjected to an array of toxic and degrading components. PMNs carry a vast arsenal of antimicrobial peptides such as  $\alpha$ -defensins, lactoferrin, cathelicidin and lysozyme pre-packed in granules (Faurischou and Borregaard 2003). These granules fuse with and release their contents into the phagosome leading to digestion of the intra-phagosomal material (Segal 2005). In addition, NADPH oxidase assembles on the phagosomal membrane generating superoxide and subsequently ROS within the phagosome. Recruitment of myeloperoxidase (MPO) to the phagosome facilitates additional enzymatic activity generating additional toxic components and chlorination of proteins (Podrez, Abu-Soud et al. 2000; Winterbourn 2002). If phagocytosis is not to occur following pathogen interaction with the PMNs, release of granule contents and ROS formation will be directed to the outside of the cell to eradicate extracellular bacteria.

Anti-microbial compounds from granules not only kill the bacteria, but may also act as chemoattractants for T-cells and iDCs (Chertov, Ueda et al. 1997; Bennouna, Bliss et al. 2003), which in turn recruit more PMNs to the site as well as initiating an adaptive immune response. Although PMNs are considered the main cells in the early phase of inflammation, this cell population has also been described to play an important role during chronic infection and tuberculosis (Kasahara, Sato et al. 1998). The role of PMNs during inflammation is much more intricate than being involved in phagocytosis and killing of microbes. PMNs has been shown to

release chemokines (IL-8, GRO $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , IFN- $\gamma$ ) and cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-12, VEGF) and additional 20 mediators have been identified to originate from PMNs and be involved in the recruitment and coordination of immune cells (Strieter, Kasahara et al. 1990; Cassatella 1995; Kasahara, Sato et al. 1998; Cassatella 1999). This gives the cell the capacity to modulate specific recruitment, regulation and orchestration of additional immune cells. Regulation of the immune response during the initial phases of infection can therefore be added to the list of PMN functions. In addition, PMNs are ascribed even more complicated roles in immunity as they shuttle bacteria from peripheral tissues to draining lymph nodes (Abadie, Badell et al. 2005). These findings are of special interest since the adaptive immune response to Mtb depends on antigen presence in the local lymph node and not in the lungs (Wolf, Desvignes et al. 2008). PMNs may thus play a major role in antigen delivery due to the fact that PMNs and DCs are present at the same site, enabling cross-talk between these cell populations. One of the more advanced properties ascribed to PMNs is their capacity to present antigens thus gaining DC characteristics (Fanger, Liu et al. 1997; Oehler, Majdic et al. 1998).

### **Innate immunity – pathogen recognition and phagocyte activation**

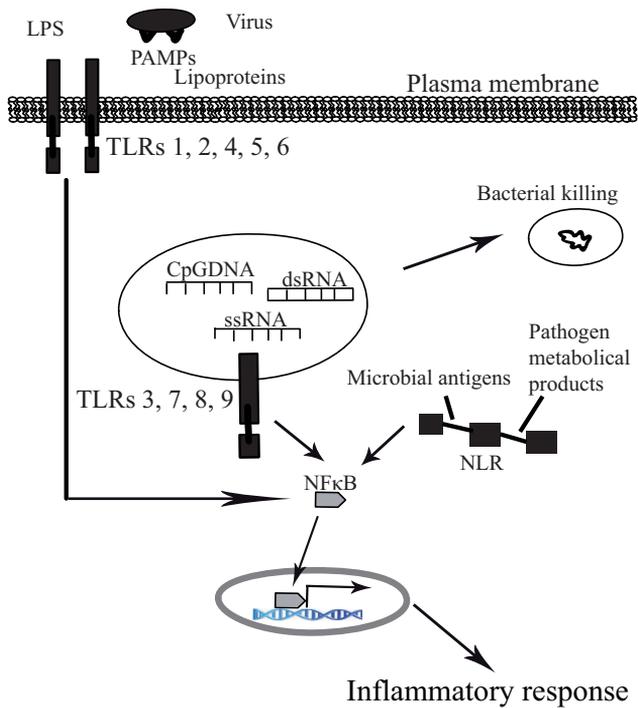
Phagocytes utilize an abundance of receptors to sample and ingest non-endogenous particles such as infectious agents. Many of these are unspecific and recognize a variety of molecular markers present on microbes, and to distinguish pathogens from other interactions, these receptors are evolutionary selected to recognize pathogen-associated molecular patterns (PAMPs) (Akira and Takeda 2004). These pattern recognition receptors are vital for macrophages and other phagocytic cells to gain information about the status of an ongoing infection by recognizing conserved motifs expressed by, or derived from microbes.

Pattern recognition receptors are constituted of two major classes called Toll-like receptors (TLRs) and NOD-like receptors (NLRs). TLRs are membrane-bound receptors, thus interacting with the extracellular or intravacuolar milieu by recognition of PAMPs including CpG DNA, LPS, lipoproteins and flagella (Medzhitov 2001). To constantly have control over the phagocytic prey, TLRs are placed along the phagocytic pathway. The TLRs are not directly

involved in the phagocytic process but rather sample presence of various microbial ligands and initiates signaling accordingly. Mitogen-activated protein kinases (MAPK) p38, ERK and JNK and various immune response genes are some of the mechanisms involved in the signaling cascade induced by TLR ligation (Akira, Takeda et al. 2001; Akira, Uematsu et al. 2006). Many of these are also tightly coupled to apoptosis induction or protection (Tran, Holmstrom et al. 2001).

TLRs 1, 2, 4, 5 and 6 are expressed on plasma membranes where they interact with the surrounding and TLR4 is abundant on early endosomes (Lawe, Sitouah et al. 2003; Chua and Deretic 2004; Kusner 2005). Activation of these TLRs promotes translocation of NfκB into the nucleus inducing pro-inflammatory cytokines such as IL-12, IL-18, TNF- $\alpha$  and IL-6 to mediate an immediate immune response to e.g. bacterial stimuli. TLRs 3, 7, 8 and 9 are present on mature phagosomes where they sample the vacuolar content following degradation of the ingested prey, thus defining the level of action needed in response to the ingested prey. Interaction with microbial material such as nucleic acid results in production and release of interferons which are key mediators in antimicrobial activities.

NODs on the other hand are soluble receptors present in the cytosol. They are thus responsible for the intracellular surveillance where they recognize DNA, RNA, cell wall components of both Gram-positive and Gram-negative bacteria and products from metabolically active bacteria. Activation of these receptors also mediates NfκB translocation to the nucleus and activates the inflammasome which is a multi-component complex (Martinon, Burns et al. 2002). The inflammasome in turn activate the central effector caspase-1 which cleave pro-IL-1 $\beta$  and pro-IL-18 into biologically active cytokines.



*Simplified model over the TLRs and NLRs. TLRs 1, 2, 4, 5 and 6 are located on the plasma membrane of the cell where they recognize diacylated lipopeptides (TLRs 2 and 6), triacylated lipopeptides (TLRs 1 and 2), LPS and various other ligands (TLR 4) and flagellin (TLR 5). TLRs 3, 7, 8 and 9 are located in the phagosomal membrane where they sample the contents for dsRNA (TLR 3), ssRNA (TLRs 7 and 8) and bacterial or viral CpGDNA (TLR 9). NLRs are located in the cytoplasm where they sample the cell for microbial antigens and products derived from microorganism metabolism. Activation of these receptors leads to translocation of NFκB into the nucleus, thereby promoting transcription of inflammatory response genes.*

## Danger signals

The immune system is however not only regulated by cell-cell interactions, pathogen recognition and cytokines but also various danger signals have been proposed to stimulate the immune response. Heat shock proteins (there are over a hundred different Hsp) have been described to be involved in a variety of cellular activities involving cell death, mitosis and

cancer development (Punyiczki and Fesus 1998; Parcellier, Gurbuxani et al. 2003; Sreedhar and Csermely 2004). Hsp (mainly Hsp60 and Hsp70) also have immuno-modulatory functions (Multhoff and Botzler 1998; Pockley 2003; Tsan and Gao 2004). The Hsp70 family comes in many shapes with the constitutively expressed 73kD Hsp70, the stress inducible 72kDa Hsp72 and the mitochondrial 75kDa Hsp75 being specific in their location as well as in function. Hsp72 was initially regarded as an intracellular chaperone protein interacting with misfolded or mutated proteins either correcting the tertiary structure or tagging them for degradation. Hsp72, has since then been shown to be a key danger signal released from a variety of cell types (Lancaster and Febbraio 2005). Hsps act through interaction with an abundance of receptors such as CD91 (Basu, Binder et al. 2001), LOX-1 (Delneste, Magistrelli et al. 2002), CD14 and TLR-2 and TLR-4 (Asea, Kraeft et al. 2000; Asea, Rehli et al. 2002) among others. The immuno-modulatory functions include inflammatory activation and enhanced phagocytic capacity and antigen presentation of macrophages (Wang, Kovalchin et al. 2006) and induction of iDC maturation (Kuppner, Gastpar et al. 2001; Somersan, Larsson et al. 2001).

Stressed human PMNs show elevated expression of Hsp60 and 72 (Eid, Kravath et al. 1987; Zheng, He et al. 2004) and even display Hsp72 on the surface (Hirsh, Hashiguchi et al. 2006) possibly through interaction with PS (Arispe, Doh et al. 2004). Taken together, these findings suggest a role for Hsp72 in the recognition of stressed or apoptotic cells by phagocytes thereby modulating the immune response accordingly.

### **Activation of macrophages**

Activation of macrophages is imperative for them to be able to engage all their anti-mycobacterial mechanisms. The most studied and important endogenous activation agents are TNF- $\alpha$  and IFN- $\gamma$ . The effects of TNF- $\alpha$  are multifaceted and enhance intracellular killing of Mtb as well as modulating the granuloma formation (Kindler, Sappino et al. 1989; Flynn, Goldstein et al. 1995; Bean, Roach et al. 1999). However, sufficient amounts of TNF- $\alpha$  must be produced since too low levels are correlated with destructive immunopathology instead of protective (Bekker, Moreira et al. 2000; Mohan, Scanga et al. 2001). The importance of TNF- $\alpha$  has been

shown in mice where sequestration of TNF- $\alpha$  in the lungs with soluble TNF- $\alpha$  receptors resulted in lethal bacterial loads (Adams, Mason et al. 1995). In addition, Mtb has been shown to induce the release of soluble TNF- $\alpha$  receptors from infected cells to inhibit the TNF- $\alpha$  based signaling of macrophages (Balcewicz-Sablinska, Keane et al. 1998). Furthermore, treatment of inflammatory disorders with anti- TNF- $\alpha$  may trigger active Tb.

T-cells, mainly CD4<sup>+</sup> has been described to exert a major role as effector cells during Mtb infections since depletion of this subset cause increase in bacterial load of persistently infected mice subsequently becoming lethal (Scanga, Mohan et al. 2000). CD4<sup>+</sup> T-cells are a major source of IFN- $\gamma$ , the hallmark cytokine involved in immune activation against intracellular bacteria such as Mtb. For instance, mice deficient in IFN- $\gamma$  are very susceptible to succumb to lethal Tb (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993). IFN- $\gamma$  stimulation of macrophages leads to NF $\kappa$ B activation affecting transcription and release of pro-inflammatory cytokines (Flynn and Chan 2001), anti-microbial measures such as NOS2 activation with NO production and upregulation of phagocytic receptors and MHC II (Anggard 1994; Boehm, Klamp et al. 1997; Collins and Kaufmann 2001; Schluger 2001) giving the macrophage an overall pro-inflammatory activation profile. Of special interest is the L-arginine-dependent, NOS2 mediated generation of NO and related RNI (MacMicking, Xie et al. 1997; Nathan and Shiloh 2000). These products are among the few described mechanism by which macrophages can kill intracellular Mtb (Chan, Xing et al. 1992) mediating toxicity at clinically relevant concentration (Nathan 2002). While the role of NOS2 is well established in the mouse with detectable levels of NO and that IFN- $\gamma$  *-/-* and NOS deficient mice are highly susceptible to Mtb (Flynn, Chan et al. 1993; Chan, Tanaka et al. 1995; MacMicking, North et al. 1997), the significance of NO in humans remains controversial (MacMicking, Xie et al. 1997). This controversy is based on the fact that there is a lack of *in vitro* culture systems able to generate NO and RNI production. Abundant expression of NOS2 has however been detected in human alveolar macrophages as well as in epithelioid macrophages and giant multinucleated cells derived from granulomatous tissue (Nicholson, Bonecini-Almeida Mda et al. 1996; Choi, Rai et al. 2002; Schon, Elmberger et al. 2004). In addition, healthy individuals living in close contact with Tb patients have been shown to have higher levels of NO production in the lungs than Tb patients (Schon, Gebre et al. 1999). However, CD4<sup>+</sup> T-cells are not necessarily the major source of IFN- $\gamma$  but the CD8<sup>+</sup> T-cells

are in fact a capable source of IFN- $\gamma$  in CD4<sup>+</sup> T-cell depleted mice (Scanga, Mohan et al. 2000). Despite the apparent production of IFN- $\gamma$ , the mice were unable to control the infection, even though NOS2 levels were normal. Thus, CD4<sup>+</sup> T-cells seems to be imperative to the control of persistent infection by a mechanism independent of IFN- $\gamma$  or NOS2 activity. Possibly, IFN- $\gamma$  and NOS2 activity are crucial in the acute phase of infection for the initial eradication of the bacteria, whereas during persistent Tb other cell-specific communications are responsible for controlling a functional granuloma. In humans, infection with HIV results in loss of CD4<sup>+</sup> T-cells leading to enhanced susceptibility to Mtb infection as well as to reactivation of latent persistent Tb.

Despite phagocytosis and onset of bactericidal events during an infection there are a few human pathogens that have specialized in surviving phagocytosis and even takes advantage of it to carry them inside a cell packed with nutrients. Intracellular microbes such as Mtb, *Salmonella typhimurium*, *Legionella pneumophila* and *Francisella tularensis* inhibit the maturation of the phagosome formed during phagocytosis. *Leishmania major* is critically dependent on the mode of the entry into the cell since one receptor-mediated uptake results in a proliferative vacuole, whereas another route of receptor-mediated uptake inevitably eradicates the pathogen (Da Silva, Hall et al. 1989). Intracellular survival is hallmarked by interfering with the fusion of lysosomes with the intracellular vacuole. Fc-receptor mediated uptake of Mtb results in survival and successful replication of the bacteria within the phagolysosomes (Flynn and Chan 2001). This failure to kill Mtb ingested by involvement of immuno-mediated Fc-receptors indicates that the adaptive immunity is insufficient to clear Mtb infections (Bhardwaj, Kanagawa et al. 1998; Feldmesser, Kress et al. 2000; Sturgill-Koszycki and Swanson 2000), a fact that further stresses that if the bacteria are to be eradicated it is essential that it is done immediately.

### **Mtb infection strategy / Modulation of the host cell**

Being an intracellular bacterium without pronounced active invasion strategies, Mtb relies on phagocytosis by the host macrophages. Mtb is recognised as a facultative intracellular pathogen that survive inside macrophages by altering the processing of the internalized bacteria-containing phagosome and inhibiting the macrophages response to IFN- $\gamma$  (Nagabhushanam,

Solache et al. 2003; Fortune, Solache et al. 2004). The failure of the phagosome to mature was originally discovered due to the observation that Mtb avoided co-localization with ferritin-labeled lysosomes (Armstrong and Hart 1971; Hart, Armstrong et al. 1972). Modulation of the phagosomal maturation starts directly during phagocytosis where Mtb interfere with the calcium flux and thereby may disturb the calcium-dependent calmodulin signaling pathway responsible for stabilizing the EEA1 interaction with PI(3)P in the phagosomal membrane resulting in failure to recruit lysosomal granules (Lawe, Sitouah et al. 2003; Chua and Deretic 2004; Kusner 2005). In addition, Mtb interfere with the phago-lysosomal fusion by inhibiting the replacement of Rab5 with activated Rab7 which is needed to provide a docking-site for the lysosome (Via, Deretic et al. 1997; Sun, Deghmane et al. 2007), retain TACO to facilitate an actin barrier around the phagosome (Ferrari, Langen et al. 1999) and inhibit intra-vacuolar acidification due to reduced incorporation of ATPase in the phagosomal membrane (Sturgill-Koszycki, Schlesinger et al. 1994; Russell 2001). This prolonged retention of early endosomal markers on the phagosome further facilitate the recycling of endosomes giving Mtb access to nutrients from the cell (Clemens and Horwitz 1996).

### **Manipulation of phagocytosis and maturation of the phagosome**

In addition to receptor interactions, the cholesterol in the plasma membrane appears to be essential for Mtb (but not other bacteria such as *E. coli*, *Yersinia pseudotuberculosis* or *S. typhimurium*) entry into both macrophages and neutrophils (Gatfield and Pieters 2000; Peyron, Bordier et al. 2000). It is however disputed whether the cholesterol is involved in direct interaction with the bacteria or if receptor interactions are dependent on cholesterol. It is however clear that many of the modulatory mechanisms described in Mtb are attributed to released or surface expressed bioactive lipids (Beatty, Rhoades et al. 2000; Russell, Mwandumba et al. 2002). In fact, Mtb subjected to surface lipid extraction is defective for survival in macrophages and such bacteria are delivered to acidified compartments (Indrigo, Hunter et al. 2003). Many of the lipids traffic throughout the vesicular compartments of the macrophage (Beatty, Rhoades et al. 2000) and the major constituent in the Mtb cell wall, lipoarabinomannan (LAM) is a highly branched carbohydrate polymer. Once shed, LAM is inserted into lipid rafts in vesicular

membranes and are affecting the fusion between the lysosome and phagosome (Shabaana, Kulangara et al. 2005; Hayakawa, Tokumasu et al. 2007; Welin, Winberg et al. 2008). LAM is recognized by TLR2 but also exert effects through interaction with host cell mannose receptors thus inhibiting the phagosomal maturation (Kang, Azad et al. 2005). The trehalose dimocylate cord factor (TDM) also display modulator functions of phagosomal maturation (Indrigo, Hunter et al. 2003; Axelrod, Oschkinat et al. 2008; Katti, Dai et al. 2008) and addition of TDM to TDM-deficient Mtb restore their ability to inhibit phagosomal maturation. It is however not known whether TDM is directly incorporation into and modulate membranes or vacuolar receptors functions in a similar fashion as LAM.

### **Modulation of signaling in the infected cell**

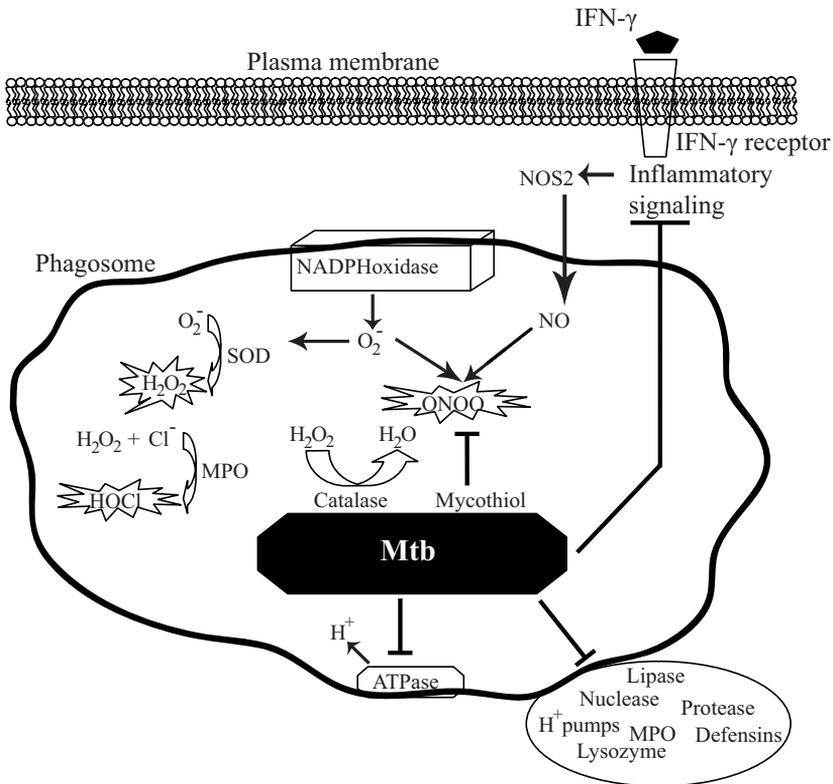
It is not only the phago-lysosomal fusion that is manipulated by Mtb. Activation of macrophages by IFN- $\gamma$  initiates mechanisms capable of handling intracellular bacteria (Collins and Kaufmann 2001) resulting in inaccessibility to carbon sources, requiring the bacteria to gain energy through long-chain fatty acid and cholesterol metabolism (McKinney, Honer zu Bentrup et al. 2000; Pandey and Sasseti 2008). To handle this threat, Mtb renders the macrophage insensitive to activation by IFN- $\gamma$  in a TLR-dependent mechanism (Kincaid and Ernst 2003; Banaiee, Kincaid et al. 2006). 19kD lipoprotein on the Mtb outer membranes is known to inhibit various macrophage activation responses to IFN- $\gamma$  (Gehring, Rojas et al. 2003; Pennini, Pai et al. 2006) and also prevent antigen presentation on MHC II thus closing down the ability to activate an adaptive immune response (Noss, Pai et al. 2001), through modulation of host cell TLR2. In addition to down-regulating the capacity of the macrophage to respond to pro-inflammatory stimuli, Mtb has been shown to modulate the macrophage to be actively immunosuppressive by inducing the production of IL-10 and TGF- $\beta$ , cytokines known to impair the ability of infected macrophages to stimulate T-cells (Hirsch, Yoneda et al. 1994; Hirsch, Hussain et al. 1996; Rojas, Olivier et al. 1999).

## Intracellular survival of Mtb

The fact that so many bacterial factors appear to be necessary for phagosomal and host cell manipulation suggests that they act in concert to create and maintain the arrested state of phagosomal maturation. However, despite the elaborate mechanisms for evading phagolysosomal fusion in the activated macrophage, it has been suggested that mycobacteria also can survive and replicate within the mature phago-lysosome. To investigate the importance of impaired phagolysosomal fusion for Mtb intracellular survival, experiments were designed to localize Mtb within a mature acidified vacuole in activated macrophages. Interestingly, despite localization of the Mtb within such mature vacuoles the investigators failed to detect any initial loss of viability of the bacteria (Schaible, Sturgill-Koszycki et al. 1998; Via, Fratti et al. 1998). This survival may be attributed to mycobacterial SOD and catalase which scavenges  $O_2^-$  and disarms  $H_2O_2$  (Harth and Horwitz 1999), mycothiol that scavenges ONOO (Newton, Arnold et al. 1996) and repair of RNI-mediated oxidative damage by methionine sulfoxide reductase (St John, Brot et al. 2001).

In addition to the modulation of and adaptation to life within the phago-lysosome, there are emerging evidence that mycobacteria do not suffice with this enclosed environment, and escapes from the vacuole and are propelled through the cytoplasm by an actin-based motility, i.e. the bacterium activates actin-polymerisation at one end thus pushing the bacterium forward in a similar fashion as *Listeria monocytogenes* and *Shigella flexneri* (Stamm, Morisaki et al. 2003). This escape from the vacuolar space is attributed the Esx-1 locus which encodes for a specialized protein secretion system (Gao, Guo et al. 2004; DiGiuseppe Champion and Cox 2007) enabling the microbe cytolytic activity and cell-to-cell spreading capacity (Myrvik, Leake et al. 1984; McDonough, Kress et al. 1993; van der Wel, Hava et al. 2007). This locus is reported to be an important virulence factor and is also found in Mtb. It has been proposed that also Mtb possess the capacity to escape from the vacuolar niche into the cytoplasm (Hsu, Hingley-Wilson et al. 2003; Guinn, Hickey et al. 2004). Although appealing to microbiologists, this theory is controversial and numerous findings state that Mtb does not gain access to the cytoplasm but are restricted to a vacuolar compartment (Clemens, Lee et al. 2002; Jordao, Bleck et al. 2008). It must however be kept in mind that most studies concerning the vacuolar niche during the first

state of infection are restricted to short study periods, and since *Mtb* is a long-lived microbe, the bacterium may however gain access to the cytoplasm later during the infection. The *Esx-1* locus is not only necessary for phagosomal escape but has been described to be involved in a calcium-dependent release of IL-1 $\beta$  and IL-18 from phagocytes (Koo, Wang et al. 2008) both known chemoattractants for phagocytes. *Esx-1* is however not necessary for the production of these cytokines since synthesis of pro-IL-1 $\beta$  and pro-IL-18 is dependent on receptor interaction (e.g. TLRs and NLRs), but *Esx-1* is only involved in the activation and release of these chemokines thus indicating activation of caspase-1. In an inflammatory perspective, IL-18 in turn induces production of MCP-1 which is a potent chemoattractant inducing degranulation of recruited NK-cells and CD8<sup>+</sup> T-cells (Loetscher, Seitz et al. 1996).



*The figure shows an Mtb within a phagosome. Phagocytic cells contract various substances to kill and degrade intracellular bacteria. Both ROS and RNIs are potent bactericidal components, which Mtb is capable of neutralizing. Mtb also inhibits additional recruitment of bactericidal peptides and enzymes and down-regulates the cells capacity to respond to IFN- $\gamma$  activation.*

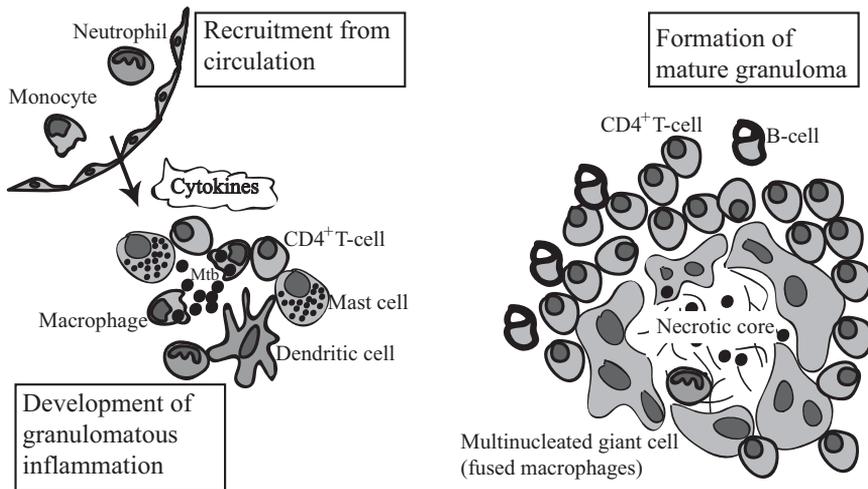
## The granuloma

When the immune system is unsuccessful in sterilizing specific infections of intracellular pathogens, such as Mtb, *Schistosoma* and *Brucella* and the microbes takes harbour inside down-regulated macrophages, the infection is enclosed within a granuloma (Cosma, Sherman et al. 2003). Latent Tb occurs when the host immune response is capable of controlling the infection but not eradicating the pathogen. Mtb has been described to enter a non-replicating state which renders them insensitive to the treatment regimens such as isoniazid which only affect replicating bacteria (Wayne and Hayes 1996). This ability to exist in the granuloma of an asymptomatic host and in a later stage reactivate into active disease is a unique feature of Mtb.

A granuloma has a characteristic morphology consisting of differentiated macrophages, T-lymphocytes, B-lymphocytes, DCs, PMNs, fibroblasts and extracellular matrix components (Adams 1976; Flynn and Chan 2001; Davis, Clay et al. 2002; Peters and Ernst 2003). The granuloma consists of a central core of necrosis (originating from the initially infected macrophages) surrounded by epithelioid cells with a peripheral cuff of lymphocytes. During persistent Tb infection, the abundance of Mtb are located in the central caseous region and to a lesser extent within macrophages constituting the granuloma (Adams 1976; Dannenberg 1993; Flynn and Chan 2001). The caseation of the granuloma is imperative for person-to-person transmission due to the rupture of the granuloma, but the mechanism underlying the formation of the liquefied caseation is unknown. Over time, granulomas can become fibrotic and calcified and these only occasionally contain live Mtb (Cosma, Sherman et al. 2003), indicating that these features of the granuloma anatomy are related to sterilized and healed infections.

The traditional view on granuloma formation is that the host uses the granuloma to confine and prevent spread of the bacteria, and individuals unable to efficiently establish granulomas succumb to active and/or disseminated Tb. In the case of Mtb granuloma, the macrophages differentiate into epithelioid cells with tight cell-cell connections to securely wall off the inside of the granuloma from the outside. Macrophages fused into giant multi-nucleated cells are often found. There are several experimental models to investigate granuloma formation and in the mouse it is shown that T-lymphocytes and other immune cells are recruited early during this

process (Flynn and Chan 2001). The bacterial growth is steadily increasing the first 2 weeks of infection whereafter the bacterial growth decline. This has been interpreted as the adaptive immunity being the determining factor for an efficient granuloma formation resulting in the halted bacterial growth. To date, all research concerning granuloma structure and function has been evaluated by resurrection and staining of fixed granulomas. This static evaluation has given rise to the notion that granuloma formation is critical for an effective restriction of bacterial growth that requires adaptive immunity to function. Recently live imaging techniques has been developed (Davis and Ramakrishnan 2009) that dramatically changes the view of granuloma formation and composition. This new technique has contributed to the understanding concerning the structure, components and immunology of the granuloma and its dynamics. Live visualisation of granuloma formation immediately led to the observation that T-cells are not constantly present at the granulomas as was previously believed, but instead come around occasionally sampling the surface of the granuloma before they move on.



*If the macrophages are incapable of inactivating or arresting growth of the ingested Mtb an immunological process, leading up to a granuloma is initiated. The granuloma is frequented by all types of immune competent cells and CD4<sup>+</sup> T-cells forms a peripheral cuff surrounding the granuloma. Macrophages fused into multinucleated giant cells are often found and the central core is caseous and contains necrotic materials and live Mtb. The image is modified from: Pathogenesis of granuloma formation, Sarraf and Sneller, Expert Reviews in Molecular Medicine 7; 8; 2005.*

## **Mtb modulates the granuloma formation**

Despite that T-lymphocytes are recruited during granuloma formation and are critical for control over bacterial growth (Andersen 1997; Saunders and Cooper 2000), it was shown in a zebrafish model with *Mycobacterium marinum* that these cells were not necessary for the initial formation of the macrophage clustering and their differentiation into epithelioid cells (Davis, Clay et al. 2002; Davis and Ramakrishnan 2009). The zebra fish model recapitulates the initial stages of the Tb infection and shows that granulomas are indeed forming within days of infection where the bacterial expansion is steadily ongoing, well before adaptive immunity is present. This model also presents an antagonizing statement to what has been described to be the essence of granuloma formation, i.e. that it is the bacteria *per se* that initiates the granuloma formation. The investigators found that monocytes from the circulation migrated to the initiated granuloma, were infected, and then themselves induced granuloma formation at a new site in the body, thus spreading the infection. These findings may explain the increased cellular adherence through LFA-1 and ICAM-1 detected in human Mtb-infected macrophages (DesJardin, Kaufman et al. 2002). Host cells of both immune and non-immune characteristics contribute to the dissemination of Mtb into systemic infection (Bermudez, Sangari et al. 2002) prior to a complete formation of granuloma. The epithelial cell barrier in the alveoli have also been described to participate in the transportation of Mtb from the lungs to other tissue both directly and by facilitating for macrophages to migrate and come in contact with the Mtb (Bermudez, Sangari et al. 2002). Also, this dissemination strategy was shown to depend on the expression of Esx-1/RD1 locus in the bacteria since bacteria deficient in this locus produced attenuated infection and failed to induce granuloma formation and recruit circulating monocytes, which is in line with the release of IL-1 $\beta$  and IL-18 from macrophages which has previously been described to be attributable to Esx-1 expressing Mtb (Mariathasan and Monack 2007; Koo, Wang et al. 2008).

Taken together, mycobacteria have the capacity to modulate measures taken by the immune cells at all stages of the inflammatory response. Mtb are capable of restricting the phago-lysosomal fusion early in infection followed by adaptation to the phago-lysosomal milieu and modulate the inflammatory response and the host cells later during the infection.

## Apoptosis

To prevent leakage of intracellular components, cells die in a strictly regulated fashion. This mechanism is called apoptosis and aged or lethally damaged cells activate an apoptosis program that facilitates the degradation and packing of cellular components. A balance between anti- and pro-apoptotic features in the cell determines apoptosis. The signaling during apoptosis is vast and new intermediates are constantly reported. Here, the basics of apoptosis induction will be described. For simplicity, the induction of apoptosis can be divided into the intrinsic and the extrinsic pathways, both adding up to cleavage of and thereby activation of pro-caspase 3 into caspase-3 which in turn executes the apoptosis (Riedl and Shi 2004). The extrinsic initiation of apoptosis originates in surface-expressed death receptors, two of which being the TNFR and FasR (Degterev, Boyce et al. 2003). The ligand for TNFR is soluble TNF secreted during inflammatory activation and the ligand for FasR is FasL expressed as a trimer on for instance committed cytotoxic lymphocytes. Upon ligation with respective ligand, the receptors cluster giving proximity for the cytoplasmic death domains that facilitates recruitment of additional components to form the death inducing signaling complex (Nagata 1997). Formation of this complex facilitates activation of the initiator caspase 8 which in turn activate caspase 3 and cleave Bid forming t-Bid, an important intermediate in the intrinsic apoptosis pathway. The intrinsic pathway of apoptosis is mediated via various mechanisms such as DNA damage, irradiation, oxidant damage, ceramide release due to e.g. TNFR ligation, all resulting in mitochondrial release of cytochrome C (CytC) and other pro-apoptotic mediators. When t-Bid complexes with Bax on the mitochondrial membrane, the electron-transport will be disturbed resulting in release of pro-apoptotic CytC and the apoptosis cascade is initiated. CytC is a crucial component in the apoptosome, a complex activating caspase 9 that in turn cleaves and activates pro-caspase 3. Caspase-3 is the main executor involving inactivation of DNA repairing systems (Casciola-Rosen, Nicholson et al. 1996; Nicholson 1996), activation of DNase and inactivation of DNase inhibitor (Sakahira, Enari et al. 1998) resulting in degradation of the DNA. In addition, inactivation of flippase, due to lack of ATP, results in increased accumulation of phosphatidylserine (PS) on the cell surface, thus providing as stable marker for early apoptotic events.

To counterbalance the pro-apoptotic signals, the cell also mobilize a variety of anti-apoptotic proteins collectively called Inhibitors of Apoptosis (NAIP, XIAP, c-IAP1, c-IAP2, survivin, livin and Ts-IAP) all exerting inhibitory functions on caspase-9, -7 and -3 (Liston, Fong et al. 2003). Due to the strict regulation of apoptosis signaling and induction, also mitochondrion-derived inhibitors such as Smac/Diablo regulate these proteins in an inhibitory fashion (Adrain, Creagh et al. 2001). In addition, non-apoptosis specific signals can exert anti-apoptotic features following cellular activation. Up-regulation and expression of MAPK, particularly ERK1/2, and PI3K/Akt pathways, inactivates caspase-9 by phosphorylation (Cardone, Roy et al. 1998) thus impairing caspase-3 activation.

### **PMN apoptosis**

The PMN is a short lived cell, (12-72 h) with a turnover rate of  $10^{11}$  cells per day. PMNs will inevitably become apoptotic due to lack of survival factors. Mcl-1 is an apoptosis-protective protein that dimerize with and prevent Bax from interacting with the mitochondrial membrane (Gardai, Hildeman et al. 2004) giving it a pivotal role in the prevention against induction of the apoptosis cascade. The lifespan can be prolonged given the right stimuli e.g. upon migration into the tissue due to integrin interaction and clustering (Berton and Lowell 1999; Whitlock, Gardai et al. 2000) and pro-inflammatory stimulation (Lee, Whyte et al. 1993). Adjacent immune cells, cytokines and engulfment of certain pathogens induce apoptosis by activation of the apoptotic cascade. The loss in structural integrity with the characteristic blebbing is the result of cytoskeleton degradation (Kothakota, Azuma et al. 1997). Due to the highly toxic and tissue degenerative contents of PMNs, a strictly controlled apoptosis and neutralization of the cell is crucial to prevent unregulated tissue destruction (Weiss 1989).

## **Pathogens modulate the apoptosis in immune cells**

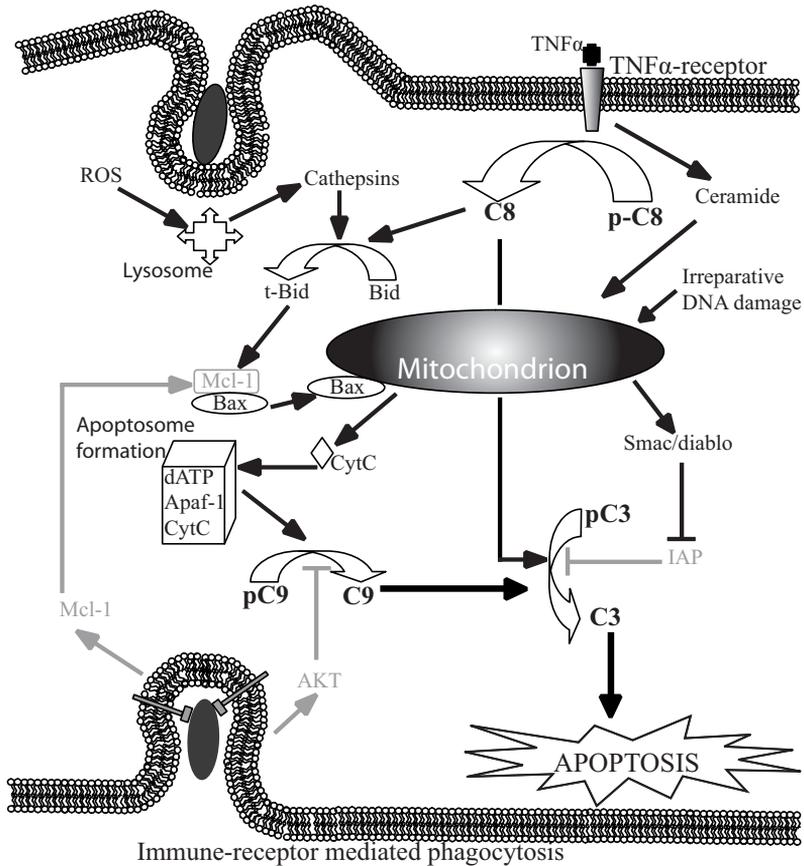
Since PMNs are packed with toxic components and the most efficient microbial killer the immune system can muster, pathogens have all to gain by inactivating this cell. The very first report on pathogen-induced apoptosis in PMNs was published in 1996 and describes how *E. coli* induce apoptosis in PMNs in a ROS dependent manner (Watson, Redmond et al. 1996). Since then, numerous reports concerning pathogen-induced cell death in PMN have been described. Although several pathogen-derived factors have been shown to delay PMN apoptosis (Moulding, Walter et al. 1999; Kim, Kim et al. 2001; Lotz, Aga et al. 2004), interaction with bacteria overrides LPS or cytokine-mediated delay on the apoptosis (Watson, Redmond et al. 1996). Pathogen-induced apoptosis in PMNs is mediated through alterations in apoptosis signaling and increased ROS production, but also genes encoding for apoptosis regulators are manipulated (Kobayashi, Braughton et al. 2003).

Being an intracellular microbe, Mtb does not only modulate the phagosomal maturation but also the lifespan of the host cell to provide a sustainable habitat. Mtb inhibits apoptosis in macrophages by activating the host cells anti-apoptotic proteins such as PI3K/Akt and MEK/ERK1/2 (Liu, Perlman et al. 2001) and also proteins involved in the suppression of the mitochondrial apoptosis pathway such as Bcl-2 (Sly, Hingley-Wilson et al. 2003). Extracellular apoptosis-signaling is prevented by inhibition of Fas-L induced apoptosis (Loeuillet, Martinon et al. 2006) and induction of cellular release of soluble TNF- $\alpha$  receptors (Balcewicz-Sablinska, Keane et al. 1998) to scavenge apoptosis-inducing TNF- $\alpha$ . Due to the capacity of PMNs to effectively kill Mtb and the short life of the cell, PMNs does not constitute a long-term harbour for slow-growing intracellular bacteria such as Mtb. Of all intracellular pathogens described it is only *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* that prolong the life-span of PMNs to provide them a host in which they replicate (Scaife, Woldehiwet et al. 2003; van Zandbergen, Gieffers et al. 2004).

In contrast to anti-apoptotic stimulation of macrophages, Mtb induce apoptosis in PMNs (Perskvist, Long et al. 2002) by phosphorylation of PLC $\gamma$ 2 with downstream activation of p38 MAPK (Perskvist, Zheng et al. 2000). Upon phagocytosis of or interaction with microbes,

PMN activate the NADPH oxidase complex and are capable of producing extensive amounts of ROS to eliminate the bacteria. However, the oxidative burst following ingestion has been described to be an essential pro-apoptotic factor in PMNs (Blomgran, Zheng et al. 2004). The production of intracellular ROS is critical for Mtb-induced apoptosis in PMNs which is executed by caspase-3 activation due to increase in pro-apoptotic Bax with simultaneous down-regulation of anti-apoptotic Bcl-XL (Perskvist, Long et al. 2002) propagating the mitochondrial pathway of apoptosis. Extensive ROS production may oxidize the membranes of azurophilic granules in PMNs (Brunk and Svensson 1999; Li, Yuan et al. 2000; Antunes, Cadenas et al. 2001). Such oxidation results in leakage of cathepsins to the cytosol and cleavage of Bid into t-Bid, propagating the mitochondrial apoptosis pathway (Blomgran, Zheng et al. 2007). The involvement of azurophilic granules and cathepsins are an effective pathway of apoptosis (Leist and Jaattela 2001; Guicciardi, Leist et al. 2004), but it is unclear whether azurophilic granules are involved in Mtb-induced apoptosis. However, during phagocytosis PMNs may be protected from apoptosis induced by pathogens. Upon immune receptor-mediated phagocytosis of opsonized bacteria, tyrosine-phosphorylation dependent signals involving Akt are activated (Forsberg, Blomgran et al. 2003; Zhang, Hirahashi et al. 2003), overriding the potent apoptosis-induction by e.g. *S. typhimurium* (Monack, Raupach et al. 1996; Forsberg, Blomgran et al. 2003) and *E. coli* (Blomgran, Zheng et al. 2004).

In addition, a clinical study showed that in PMNs from patients with active tuberculosis, the apoptosis was increased upon interaction with Mtb (Aleman, Schierloh et al. 2004). The authors concluded that the increased apoptotic response was dependent on p38 activation and bacterial interaction with TLR2. It was however only the bacteria that induced the increased apoptosis and not cell wall fraction or soluble LAM, indicating that additional receptor interactions and cross-activation may be involved.



*Apoptosis signaling involves an abundance of mediators and inhibitors. The figure shows a simplified model over involved signals during apoptosis in neutrophils. The mitochondria is important in the signaling cascade with release of both inhibitory and apoptosis-activating mediators. ROS production following pathogen interaction, uptake or invasion can damage lysosomal membranes leading to release of cathepsins, which activate t-Bid, in turn affecting CytC release from the mitochondrion. Immune-receptor mediated uptake of bacteria induces Akt and Mcl-1 that protects from apoptosis due to the inhibition of caspase-9 activation. C= caspase, IAP= inhibitors of apoptosis. Black pathways = pro-apoptotic and grey pathways = anti-apoptotic.*

## Clearance of apoptotic cells

The term apoptosis was originally coined by Kerr and colleagues (Kerr, Wyllie et al. 1972; Wyllie, Kerr et al. 1980) and Metchnikoff was first to describe the removal of apoptotic cells by phagocytes in inflamed tadpole fins in a lecture at the Pasteur Institute in 1891 (Newman, Henson et al. 1982). It has since then been established that clearance of apoptotic cells is crucial to avoid leakage of intracellular contents to prevent extensive tissue destruction. Apoptotic cells display changes in the plasma membrane early after initiated apoptosis with loss of symmetry and exposure of PS and a multitude of apoptotic markers on the outer leaflet of the plasma membrane. These events and exposed apoptosis-specific ligands are crucial for the recognition and removal of the apoptotic body by phagocytes (Fadok, Voelker et al. 1992; Gregory and Devitt 2004) a mechanism that has been described to be immunologically silent or anti-inflammatory. Macrophage uptake of apoptotic cells is in a sterile environment associated with down-regulation of inflammatory cytokines such as GM-CSF, TNF- $\alpha$ , IL-12 and IL-1 $\beta$ , and up-regulation of the anti-inflammatory cytokines e.g. TGF- $\beta$ , PGE2 and PAF (Voll, Herrmann et al. 1997; Fadok, Bratton et al. 1998), resulting in an overall anti-inflammatory reaction.

Since apoptosis is the inevitable fate of PMNs, they are cleared by neighbouring cells. In 1998 Fadok et al showed that ingestion of UV-induced apoptotic PMNs induced an anti-inflammatory cytokine production in macrophages presenting the idea that inflammatory resolution is mediated by apoptotic cell uptake (Fadok, Bratton et al. 1998). Involvement of apoptotic cells in the resolution of inflammation has since then been shown over and over again. UV-induced apoptotic PMNs even have the capacity to down-regulate the inflammatory response induced in macrophages by LPS and zymosan (Fadok, Bratton et al. 1998). The importance of apoptotic cell clearance have also been described in clinical settings with SLE patients, who develop autoimmunity to intracellular antigens due to leakage from non-cleared post-apoptotic cells (Herrmann, Voll et al. 1998). DC interaction with apoptotic cells can induce tolerance to peripheral self antigens which may be a missing link in the SLE patients.

## **Ligands, receptors and bridging molecules involved in apoptotic cell removal**

Interaction with and uptake of apoptotic bodies involve several receptor–ligand interactions as well as bridging molecules (Ren and Savill 1998; Maderna and Godson 2003; de Almeida and Linden 2005; Henson and Hume 2006). This abundance of ligands and receptors indicates that the interaction-site between the cells is riddled with multiple low-affinity interactions similar to the immunological synapse, described between T-cells and antigen-presenting cells, and will therefore be discussed as a phagocytic synapse. The interactions between the phagocyte and the apoptotic cells are generally divided into “eat me” and “don’t eat me” signals due to their capacity to either induce or inhibit apoptotic cell uptake. Four distinct phases from the initial recognition to the phagocytosis are distinguished; recognition (inter-cellular interaction determining if a phagocytic synapse should be formed), tethering (binding and formation of the phagocytic synapse), signaling (initiation of engulfment and production of anti-inflammatory mediators) and subsequently engulfment.

### **Eat me**

PS is the main early membrane marker for apoptotic cells. The interactions investigated with PS are vast, involving scavenger/pattern-recognition receptors CD14, CD36, CD68, LOX-1, vitronectin receptor and the specific receptor for PS (PSR), all of them triggering immunosuppressive mechanisms (Savill, Hogg et al. 1991; Sambrano and Steinberg 1995; Devitt, Moffatt et al. 1998; Oka, Sawamura et al. 1998; Fadok, Bratton et al. 2000). However, Barth’s syndrome is a condition where circulating PMNs continuously expose PS, but without being cleared by macrophages (Kuijpers, Maiani et al. 2004). This indicates that exposure of PS *per se* is not sufficient for engulfment. Oxidation of PS has been proposed to be essential for the recognition by phagocytes to be initiated. For instance, CD36 recognizes only the oxidized but not non-oxidized PS (Greenberg, Sun et al. 2006). In addition, oxidation of PS is a plausible feature since lipid peroxidation increases in an inflammatory milieu.

The diversity of the apoptotic cell plasma membrane has encouraged investigators to explore the interactions in the phagocytic synapse in detail. Stressed cells expose calreticulin on the surface (Heal and McGivan 1998; Ogden, deCathelineau et al. 2001), a ligand that interact with the phagocyte receptor LRP/CD91 as part of the receptor complex involved in the phagocytic synapse, activating the Rac-1 GTPase involved in the engulfment of apoptotic cells (Gardai, McPhillips et al. 2005). However, surface expression of calreticulin is not specific for apoptotic cells and to be recognized as an “eat me” marker, calreticulin must be displayed in association with PS. Exposed annexin-1 on the apoptotic cell stimulates IL-10 production (Ferlazzo, D'Agostino et al. 2003), which in turn exert autocrine and paracrine anti-inflammatory effects, and enhance phagocytosis of apoptotic cells by macrophages (Ogden, Pound et al. 2005). Additionally, anti-inflammatory lipoxin shown to be formed due to transcellular metabolism between PMNs and thrombocytes (Serhan and Sheppard 1990), potentially increase macrophages uptake of apoptotic cells (Mitchell, Thomas et al. 2002). Apoptotic PMNs will thus actively facilitate increased uptake and subsequent resolution of inflammation.

### **Don't eat me**

CD31 is present on both viable and apoptotic cells but it is only the disabled CD31 on apoptotic cells that mediate the selective engulfment of the apoptotic cell, since native CD31 signals in a “don't eat me” fashion (Brown, Heinisch et al. 2002). In addition to native CD31, exposure of CD47 on live cells inhibit engulfment upon interaction with SIRP $\alpha$  on the phagocyte (Oldenborg, Zheleznyak et al. 2000; Gardai, McPhillips et al. 2005). This is proposed be the result of recruited phosphatases that dephosphorylate tyrosine-dependent receptors thus inhibiting their signaling (Kharitononkov, Chen et al. 1997).

### **Bridging molecules**

It is however not only direct ligand-receptor interactions that are involved in the phagocytosis of apoptotic cells. An abundance of extracellular bridging molecules, for instance thrombospondin that interact with CD36 (Savill, Hogg et al. 1991; Savill, Hogg et al. 1992) and

complement components increase the phagocytosis of apoptotic neutrophils by macrophages (Mevorach, Mascarenhas et al. 1998). Thus, the interplay between apoptotic cells and phagocytes appears to create a microenvironment that not only suppresses immune and inflammatory responses but also facilitates efficient apoptotic cell clearance.

### **Uptake of apoptotic PMNs – not only resolution of inflammation**

The notion of apoptotic cells are important to the resolution of inflammation is widely accepted. When pathogens are removed, the surplus of recruited PMNs will, in the absence of microbes, become spontaneously apoptotic and upon clearance by macrophages induce anti-inflammatory resolution of the inflammation. It is evident that an effective inflammatory response is crucial to handle infections but to avoid extensive tissue leakage and a chronic inflammatory state, the resolution of the inflammatory response is imperative. However, many investigators are excluding an actual cause of the inflammation, i.e. the infectious agent and have studied the effects of uptake of apoptotic cells in sterile models. Recently, it was shown that pathogens such as *Leishmania major* invade PMNs, induce apoptosis and then silently enter the macrophage in a non-immuno activation fashion through apoptotic cell clearance thus facilitating the parasites' intracellular survival (van Zandbergen, Klinger et al. 2004; Ribeiro-Gomes, Moniz-de-Souza et al. 2005). This finding is somewhat contradicted by the fact that engulfment of bacteria- and virus-infected apoptotic cells by DCs results in presentation of pathogen-specific antigens (Larsson, Fonteneau et al. 2001) and *S. typhimurium*-induced apoptotic PMNs activate the macrophages that clear them (Zheng, He et al. 2004). In addition, macrophages have been shown to be dependent on antibacterial peptides delivered from apoptotic PMNs to be able to kill intracellular bacteria such as Mtb (Tan, Meinken et al. 2006). Disputing data taken aside, it is however clear that a strict down-regulation of inflammatory activation in the presence of bacteria would be detrimental to the host. If the anti-inflammatory properties were as potent as they are described to be, intracellular bacteria (and extracellular not yet phagocytosed) would thrive as soon as PMNs became apoptotic and induced anti-inflammation in the macrophage population.



## Results and Discussion



## **Results and discussion**

### **What are then the aims and hypothesis of this work?**

PMNs and macrophages are important effector cells in innate immunity with the main focus on phagocytosis and intracellular killing of pathogens. In the interaction with intracellular pathogens, such as Mtb, PMNs are killed and macrophages become a safe haven for the bacterium. Subsequently, apoptotic PMNs containing Mtb are recognized and cleared by macrophages. This raised questions whether apoptotic PMNs could influence how infected macrophages handle intracellular Mtb at the early phase of the infection. We therefore investigated **i**; how Mtb induce apoptosis in PMNs, **ii**; how these apoptotic cells in turn affect macrophages and DCs upon clearance and what this means to the infection status.

In this work we have used human material such as isolated peripheral PMNs, and macrophages and DCs derived from peripheral blood monocytes. All cells were isolated from whole blood or buffy coats.

### **Interaction with Mtb induce apoptosis in PMNs**

PMNs effectively kill and degrade phagocytosed preys but interaction with some pathogens such as Mtb promotes apoptosis. We therefore investigated the nature of Mtb-induced apoptosis in PMNs. Phagocytosis of different prey has been described to affect the apoptotic outcome in PMNs and we investigated whether the phagocytosis of Mtb was required for the bacteria to induce apoptosis in PMNs. Unopsonized Mtb were neither phagocytosed nor were they attached to the PMNs, whereas serum-opsonized Mtb were readily phagocytosed by PMNs. Despite this difference in interaction between the bacteria and phagocyte, both unopsonized and opsonized Mtb induced apoptosis to a similar extent. This indicates that the signal for induction of apoptosis is derived by surface interaction and does not require uptake of the Mtb.

Since non-phagocytosed Mtb potentially induced apoptosis in PMNs we investigated possible surface expressed virulence factors capable of this induction of apoptosis. Mycobacterial 19kD lipoprotein in suspension has been shown to activate PMNs (Neufert, Pai et al. 2001) and induce apoptosis in macrophages (Lopez, Sly et al. 2003). Using a mutant Mtb-strain deficient in lipoprotein signal peptidase (*lspA*<sup>-/-</sup>), rendering the bacteria incapable of posttranslational modification of lipoproteins, allowed us to investigate the involvement of mature lipoproteins in the induction of PMN apoptosis. We found that bacteria deficient in mature lipoprotein were incapable of inducing apoptosis in PMNs. Despite the fact that opsonized mutant bacteria were phagocytosed to the same extent as Wt Mtb, no induction of apoptosis occurred further indicating that induction of apoptosis is triggered by the initial interaction with the PMN. Our finding that the *lspA*<sup>-/-</sup> mutant did not provoke apoptosis supports the view that mature lipoproteins are necessary for induction of apoptosis also in PMNs as has previously been described in macrophages using recombinant 19kD lipoprotein. To clarify the involvement of mature lipoproteins we subjected the PMNs to the *lspA*<sup>-/-</sup> mutant together with a triacylated peptide (Pam<sub>3</sub>CysSK<sub>4</sub>) mimicking the acylations on mature 19kd lipoprotein. This treatment restored the apoptosis to the same level as Wt bacteria. Pam<sub>3</sub>CysSK<sub>4</sub> did not exert any direct effect on PMN apoptosis but merely restored the apoptosis inducing potential of the *lspA*<sup>-/-</sup> Mtb.

Mtb-induced apoptosis in PMNs is dependent on ROS production by NADPH oxidase (Perskvist, Long et al. 2002) and we found that treatment of PMNs with a NADPH oxidase inhibitor (DPI) abrogated the apoptosis. To further understand the involvement of ROS we measured the production and localization of ROS in PMNs following exposure to Mtb. Opsonized Mtb mainly induced intracellular ROS whereas unopsonized Mtb induced both intracellular and extracellular ROS to a similar extent. The total amount of produced ROS was similar despite the difference in localization. However, the intracellular ROS was crucial to the induction of apoptosis since removal of extracellular ROS (by presence of SOD and catalase) only affected the apoptosis to a minor extent. Nevertheless, the formation of ROS was quite limited (about 10%) as compared to levels induced by other apoptosis-inducing pathogens such as FimH<sup>+</sup> *E. coli*. The fact that the limited ROS response was prolonged and did not reach basal level until after 10 h, suggests that Mtb-induced ROS production have signaling properties at

nontoxic concentrations as has been described (Gamaley and Klyubin 1999), rather than serving as an executor of apoptosis.

However, the *lspA*<sup>-/-</sup> strain, lacking mature lipoproteins, induced similar amounts of ROS as Wt bacteria, but failed to induce apoptosis, indicating that ROS is not the sole trigger of apoptosis, but additional Mtb-specific signals are required.

The mitochondria are often involved in the induction of apoptosis due to release of CytC necessary for formation of the apoptosome and activation of effector caspases. We investigated whether the mitochondria were affected in Mtb-induced apoptosis in PMNs by measuring the mitochondrial membrane potential. This technique monitors how well the mitochondria can shuttle electrons over the membrane, and a lack in this potential indicates that mitochondria are involved in the initiation/promotion of the apoptosis. It can however be argued that since PMNs contain few mitochondria, these organelles are of minor importance in the cell. The mitochondrial pathway of apoptosis was however activated following interaction with Mtb, which is supported by previous observations showing that expression of antagonizing Bcl-2 proteins is altered early during Mtb-induced apoptosis (Perskvist, Long et al. 2002).

Intracellular ROS such as H<sub>2</sub>O<sub>2</sub> oxidizes lipids in granule membranes, which results in leakage of cathepsins from the vacuole and activation of the mitochondrial apoptosis induction pathway (Roberg, Johansson et al. 1999; Antunes, Cadenas et al. 2001). Due to the fact that phagocytosis of Mtb occurs without fusion of azurophil granules, and therefore renders the PMNs with large numbers of unfused granules (Perskvist, Roberg et al. 2002), we investigated whether the ROS production during Mtb-induced apoptosis was dependent on oxidation and subsequent enzyme leakage from azurophil granules. We did not detect any labilization of the lysosomal membrane during Mtb-induced apoptosis. This indicates that the lysosomal damage is not crucial for the induction of apoptosis, and that the mitochondrial pathway of apoptosis is induced by another pathway.

## **Signaling during Mtb-induced apoptosis.**

Uptake of serum-opsonized particles mediated mainly through CR3 triggers both pro- and anti-apoptotic signals. NADPH oxidase activation on one side enhances apoptosis, whereas CR3-mediated uptake on the other activate anti-apoptotic actions through MAPK/ERK activation and PI3K/Akt dependent pathways involving increased Mcl-1:Bax interaction thereby decreasing caspase activation. Immune receptor-mediated phagocytosis of virulent bacteria has been shown to induce anti-apoptotic proteins (Akt and MAPK/ERK) and reduce apoptosis (Forsberg, Blomgran et al. 2003; Zhang, Hirahashi et al. 2003). Phagocytosis of opsonized Mtb induced early and sustained upregulation of Akt, but despite this, the apoptosis induced by the bacteria was not reduced as detected by inhibition of Akt activation. This indicates that Mtb has a strong intrinsic potential to induce apoptosis in PMNs and is able to overcome the anti-apoptotic Akt-dependending mechanisms following immune-receptor mediated phagocytosis.

Mtb-induced apoptosis in PMNs have previously been described to be dependent on upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-xL (Perskvist, Long et al. 2002). We found that Mtb-induced apoptosis in PMNs was critically dependent on activation of p38 and the apoptosis was abrogated by inhibiting the activation of this signal. These data are supported by the finding that PMNs in Tb patients show elevated levels of p38 and that these cells are also more prone to become apoptotic than healthy control cells (Aleman, Schierloh et al. 2004). It is however likely that PMNs come in contact with the Mtb in tissues, and it is unlikely that they would migrate out to the blood again, especially since we have shown that they enter apoptosis quite rapidly. The study could thus show that the general inflammatory state in Tb patients with elevated levels of TNF- $\alpha$ , which have pro-apoptotic properties on PMNs, is the reason for such findings in Tb patients. It would however be of great interest to investigate presence of mycobacterial material in these patients' PMNs, or at least determine the inflammatory state and cytokine levels in these patients.

## **Clearance of apoptotic PMNs.**

Apoptotic cells are cleared by neighbouring phagocytes and even non-phagocytic cells. This clearance has been described to be immunologically silent with release of anti-inflammatory cytokines by phagocytosing macrophages. Since Mtb have the capacity to induce apoptosis in PMNs we wanted to investigate whether these Mtb-induced apoptotic cells were cleared in the same fashion as are aged apoptotic cells. We found that Mtb-induced apoptotic PMNs and spontaneously apoptotic PMNs were cleared to the same extent by macrophages. In contrast, Mtb-infected apoptotic PMN elicited a potent pro-inflammatory response in macrophages with the release of pro-inflammatory TNF- $\alpha$ .

To investigate the features responsible for this fundamental difference in the response to Mtb-induced apoptotic PMNs we measured the levels of the stress or danger signaling molecules Hsp. Such proteins have been described to exert potent activation of immune cells and we found that presence of recombinant Hsp70 or Hsp60 synergistically augments the Mtb-induced activation of macrophages. We further found that PMNs upregulated Hsp72 and Hsp60 early after exposure to Mtb whereas spontaneously apoptotic PMNs did not express any amount of neither Hsp60 nor Hsp72.

In addition to intracellular upregulation of Hsp72 we found that Mtb-induced apoptotic PMNs also released the protein via an exosome-like mechanism. Hsp60 being a mitochondrion affiliated protein was not released from the apoptotic PMNs. The exosomal release of Hsp is supported by findings described during IFN- $\gamma$  stimulation of specific cancer cells (Bausero, Gastpar et al. 2005). The release was not the result of unspecific leakage of cellular contents from the apoptotic cells, since the cytosolic enzyme LDH was not present in the culture medium. The expression of Hsp was initiated by phagocytosis of the bacteria rather than the subsequent apoptosis process, since the expression of Hsp60 and Hsp72 was not ROS dependent, and caspase inhibition did not affect the release.

Notably, released Hsp72 in contrast to the intracellular upregulation of this protein was dependent on ROS. We further found that Hsp72 containing culture media from Mtb-induced apoptotic PMNs exerted no direct effect on macrophages but instead strongly inflicted a synergistic augmentation of the pro-inflammatory response to low numbers of Mtb in a similar fashion as did recombinant Hsp70. This indicates that both the Mtb-induced apoptotic PMNs *per se* as well as released Hsp72 modulate macrophages in a pro-inflammatory manner.

Hsp72 has been shown to interact with PS, and could therefore modulate the macrophage recognition of and response to apoptotic cells by changing the response from an anti-inflammatory to a pro-inflammatory activation. We did however not detect any effect on macrophage activation, by apoptotic PMNs pre-incubated with Hsp70, thus indicating that if coupling to PS is to occur it may do so before PS is exposed on the cell surface.

Taken together, our observations suggest that, Mtb-induced apoptotic PMNs, besides killing Mtb, activate macrophages thus helping them to eradicate rather than confiscate phagocytosed Mtb. The recent observation that Mtb can trigger local tissue macrophages to further attract peripheral cell infiltration (Davis and Ramakrishnan 2009) supports the view that tuberculosis is a complex infection affecting not only the local immune response, but cell traffic as well. Since Hsp60 and Hsp72 has been proposed to be ligands for TLRs, the activating signals may originate from this interaction, since these receptors function as modulators of the innate immune response and inflammation (Sabroe, Read et al. 2003). In addition to stimulating pro-inflammatory cytokine response, TLRs has been found to regulate phagolysosome maturation and subsequent inactivation of the ingested prey (Blander and Medzhitov 2004). Whether interaction between Hsp72 and TLRs occur during cell-cell contact or during sampling of intraphagosomal contents is however not clear.

Mtb will induce maturation of iDC leading to activation of the adaptive immune response (Tian, Woodworth et al. 2005) giving DCs a pivotal role in the establishment of immunological memory against Mtb. Since DCs also recognize and ingest apoptotic cells, we wanted to evaluate how Mtb-induced apoptotic PMNs affected DC maturation. We found that spontaneously apoptotic PMNs did not induce DC maturation whereas Mtb-induced apoptotic

PMNs were able to induce DC maturation following 48 h co-culture. Mature DCs in turn stimulated T-cell proliferation showing that they were competent to induce expansion of T-cells, indicating this potency to activate an adaptive immune response. Both spontaneously apoptotic PMNs and Mtb-induced apoptotic PMNs, bound to the DCs, but only few cells were actually internalized by DCs. This is supported by the finding that apoptotic PMNs are phagocytosed by iDC only when presented in vast excess (Clayton, Prue et al. 2003). Maturation mediated by Mtb-induced PMNs was however inhibited by cytochalasin D, indicating that endocytosis is important. We can therefore not rule out that smaller apoptotic blebs or plasma membrane from the adherent PMNs were nibbled off and internalized by the iDC (Albert, Sauter et al. 1998; Harshyne, Zimmer et al. 2003). We further found that the Mtb-induced apoptotic PMNs induced maturation was dependent on surface interaction between the Mtb-induced apoptotic PMNs and DC. Resting and LPS-activated PMNs bind to iDCs through a glycosylation-dependent binding between Mac-1 expressed on the PMN and the DC-specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN) on the DC surface (van Gisbergen, Ludwig et al. 2005). We found that Mtb-induced apoptotic PMNs mediated maturation of DCs was dependent on the interaction between the Mac-1 on the apoptotic cell and DC-SIGN on the DCs. By inhibiting this interaction with either anti DC-SIGN antibodies or denatured albumin, thereby occupying the Mac-1 (Davis 1992), maturation was abrogated. However, the integrin-dependent activation of DCs was not a result of either increased affinity formation or general upregulation on the Mtb activated PMNs. This indicates that involvement of Mac-1 may be dependent on co-localization with other stimulatory molecules on the Mtb-induced apoptotic cell (such as PS or possibly Hsp72) rather than an increase in expression or affinity.

Hsp can induce DC maturation (Banchereau and Steinman 1998; Somersan, Larsson et al. 2001), but Hsp72 released from the Mtb-induced apoptotic cell did neither directly affect the maturation of the DC nor did presence of Hsp72 augment Mtb-induced maturation of the DCs. We therefore conclude that exogenously delivered extracellular signaling is not crucial for crosstalk between Mtb-induced apoptotic PMNs, suggesting that maturation truly requires a cell-cell interaction and subsequent endocytosis.

### **Apoptotic PMNs – more to the story than resolution of inflammation.**

Uptake of spontaneously apoptotic PMNs induce anti-inflammatory mechanism in the clearing macrophage with release of anti-inflammatory cytokines and down-regulation of pro-inflammatory cytokines. Since there is a massive infiltration of PMNs to the site during inflammation, it is most likely that these short lived cells will be present both as active viable and as apoptotic cells at the same site as the bacteria. During inflammation, release of e.g. IL-12 (data not shown) act on the *de novo* production of PMNs in the bone marrow, leading to even more recruited cells. The massive influx of PMNs at the infected site is described to aid in the removal of bacteria, whereafter the apoptotic PMNs mediate in the resolution of inflammation due to their potent anti-inflammatory interaction with macrophages.

To mimic the inflammatory setting during infection we exposed macrophages to both apoptotic PMNs and bacteria simultaneously. We found that in the presence of bacterial stimuli, apoptotic PMNs exerted the opposite immune regulatory functions than has previously been described. In fact, in the presence of apoptotic PMNs the macrophages TNF- $\alpha$  release in response to either Mtb or *S. typhimurium* increased substantially. It can be argued that the effect may be dependent on two prey of different size being ingested simultaneously, thus involving a multitude of interactions that together result in the increased activation. We however found that the order in which we added the two preys to the macrophages did not matter. This indicates that the anti-inflammatory modulation that apoptotic PMN exert alone on the macrophages is not definite, since subsequent addition of bacteria produces a stronger inflammatory response in the macrophages than the bacteria alone. To investigate whether the effect was mediated by the mere phagocytosis of a large prey, we substituted the apoptotic PMNs with latex beads of the same size. This treatment did however not modify the response to the bacteria.

Not only the cytokine secretion was enhanced but the killing capacity of Mtb as well. Since Mtb survive inside macrophages, and are able to inhibit the macrophage from being activated by IFN- $\gamma$  (Ting, Kim et al. 1999), apoptotic PMN could initiate a back-up system capable of activating the macrophages at the early stage of infection.

Secreted TNF- $\alpha$  from activated macrophages also exert a direct effect on PMNs since it enhances their bactericidal activity (Knowles, Keeping et al. 1997). Presence of activated macrophages further induce apoptosis in adjacent PMNs, possibly through released or surface expressed TNF- $\alpha$  (Allenbach, Zufferey et al. 2006). TNF- $\alpha$  from activated macrophages could therefore enhance both the efficiency of the PMNs to kill Mtb and facilitate that infiltrating PMNs enter apoptosis. In the presence of microbial stimuli, the capacity of macrophages to kill bacteria as well as further recruit immune cells would be increased by the apoptotic PMNs as long as the infection persists.

Our data together with the observation that PMN ingest *Mycobacterium bovis* and then migrate to the lymph nodes (Abadie, Badell et al. 2005), imply that PMN play a more extensive role in the regulation of mycobacterial infections since immunity to Mtb has been described to be initiated in the local lymph nodes and not in the lung (Wolf, Linas et al. 2007).



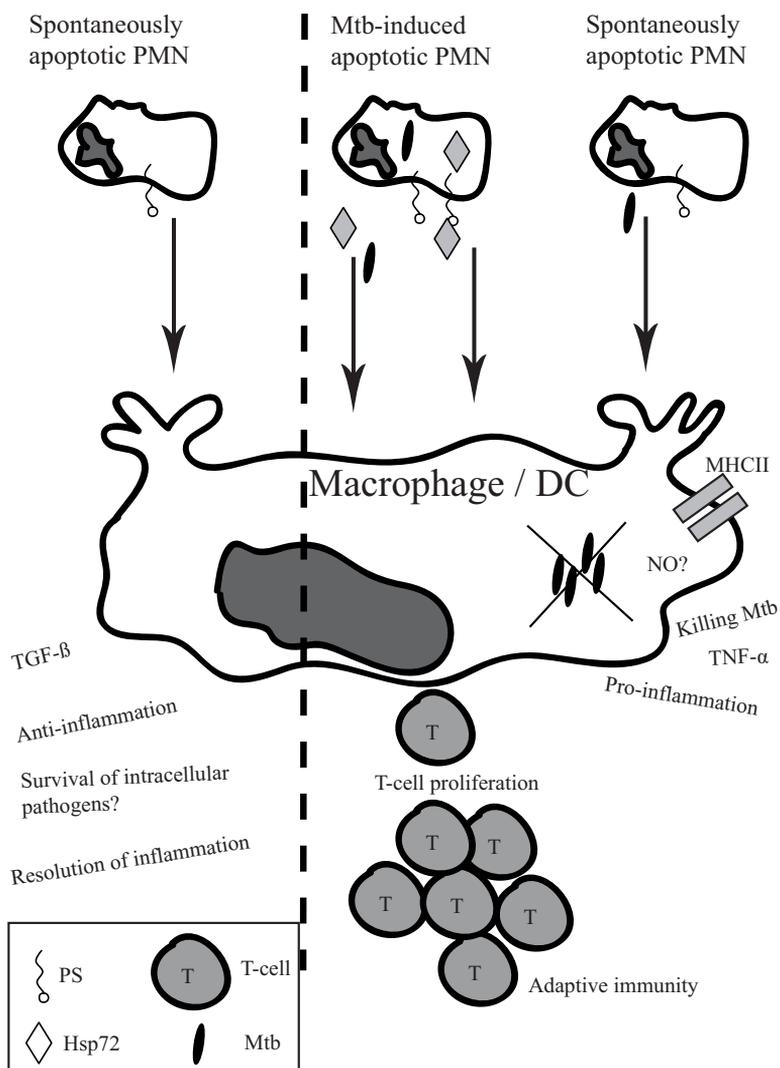
## Concluding remarks



## **Concluding remarks**

The main reason for our research on Mtb is the fact that only a fraction of people exposed to Tb become infected and even fewer become sick. This indicates to us that the immediate response must be performed by innate immune cells but whether these individuals acquire immunological memory is not known. We have shown that in the presence of Mtb, apoptotic PMNs activate both macrophages and DC and propose that this is important for an efficient and immediate immune response. Taken together, PMNs not only kill their share of Mtb while living, they also modify the remaining cells and increase their capacity to battle Mtb infections when they die. We hypothesize that this inflammatory activation mediated by apoptotic PMNs presents a link between the early innate immunity and the adaptive immunological memory during infections.

The fact that apoptotic PMNs can enhance macrophage and DC functions against Mtb may offer a new model for development of vaccines and adjuvant treatments. To be able to develop specific targets, the main challenge is to understand how macrophages and DCs recognize and discriminate between different apoptotic cells.



*Interaction of apoptotic PMNs with macrophages and DCs. Left; uptake of spontaneously apoptotic PMNs by macrophages or DCs does not inflict inflammation but is involved in the resolution of inflammation. Center left; released Hsp72 from Mtb-induced apoptotic PMNs induce pro-inflammatory activation of macrophages in response to Mtb. Middle right; Mtb-induced apoptotic PMNs induce a pro-inflammatory activation of macrophages and induce maturation in DCs enabling them to stimulate T-cell expansion. Left; spontaneously apoptotic PMNs inflict a potent pro-inflammatory activation in macrophages in response to Mtb and enhance the capacity of Mtb-infected macrophages to kill intracellular Mtb.*

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