Differential Influence of Clinical *Mycobacterium avium* Strains on Distinct Autonomous Defense Capacities of Human Neutrophils

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To the love of my life....

“host-pathogen interaction and the evolutionary arms race”

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1 Introduction

1.1 Mycobacteria

Mycobacterial pathogens present a health threat worldwide with *Mycobacterium tuberculosis (Mtb)* alone accounting for 1.3 million deaths and 8.6 million new and relapse infections in 2012 (http://www.who.int/tb/publications/global_report/en/). Tuberculosis is an infectious bacterial disease caused by the facultative intracellular pathogen *Mycobacterium tuberculosis* that is still posing a major health and socio-economic burden at a global level, primarily in low and middle-income countries. The genus *Mycobacterium* includes more than 60 species and more than 100 sub-species (http://www.dsmz.de). Currently a number of 8 species are referred to as the *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis (including the BCG strain)*, *M. africanum*, *M. microti, M. canettii, M. caprae, M. pinnipedii, M. mungi*) and all of these can cause the clinical picture of tuberculosis. *M. tuberculosis and M. leprae* were recognized as pathogenic for humans only about 140 years ago and their pathogenic behaviors have been studied extensively. Mycobacteria other than *M. tuberculosis* and *M. leprae*, are called non-tuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT) and about 20 species of them cause human and animal diseases (Heifets, 2004). NTM species are ubiquitous in the environment, predominantly populating soil and water (Falkinham, 2010, 2002; Taylor et al., 2000). They have been recovered from surface water, tap water, soil, domestic and wild animals, milk, and food products (Thomson et al., 2007). The incidence of infection and disease due to non-tuberculous mycobacteria (NTM) is rising in many countries. It was thought that NTM are more prevalent in tropical countries (Africa and Australia), however, accumulation of recent data suggests that it is also prevalent in temperate countries like USA, Canada, Germany, United Kingdom and China (Behr, 2008; Thomson, 2010).

NTM and specifically *M. avium* became inevitably recognized as pathogens causing severe diseases in humans at the beginning of the HIV/AIDS era. Back then *M. avium* caused severe, mostly fatal infections in immuno-compromised patients. The most prominent risk factors for the development of disseminated *M. avium* infection were a CD4 cell-count of less than 100/ml and previous colonization of mucosal surfaces with *M. avium* (Horsburgh,

1991; Klatt et al., 1987; Mapother and Songer, 1984; Nightingale et al., 1992). Although there is a strong correlation between *M. avium* disease and low CD4 cell-counts, a specific immune defect facilitating invasion and dissemination of *M. avium* has not yet been defined. Uptake and intracellular growth inhibition of *M. avium* by monocytes derived from AIDS patients has been shown to be normal in vitro. Moreover, the application of granulocyte colony stimulating factor G-CSF, a cytokine that specifically activates neutrophils was beneficial with regard to survival of AIDS patients suffering from *M. avium* infection (Keiser et al., 1998). This implicates that neutrophils may have a protective role in *M. avium.* Moreover, there are reports showing that *M. avium* and other NTM cause lung infection (also called atypical mycobacterial pulmonary infections) in immuno-competent individuals (Piersimoni and Scarparo, 2008; Taiwo and Glassroth, 2010). Also, epidemiologic surveillance reveals that the occurrence of NTM diseases is increasing in industrialized nations where TB transmission is low and where BCG vaccines are not given (Lietman et al., 1997; Winthrop et al., 2010). The incidence of NTM infections is particularly increasing in the elderly (Mirsaeidi et al., 2014), most of them exhibiting pulmonary infection caused primarily by *M. avium*. Some of these lung infections occur as a complication of pre-existing lung disease, such as bronchiectasis and chronic obstructive pulmonary disease. However, some other infections occur in healthy hosts without pre-existing lung disease or overt immunologic disorder (Huang et al., 1999; Koh et al., 2002; Marras et al., 2013; Sadek et al., 2008; Thomson, 2010). Generally, non-tuberculous mycobacteria (NTM) inhabit body surfaces and secretions without causing disease. However, under certain conditions, which are not fully understood, they can induce some clinical manifestations such as progressive pulmonary disease, superficial lymphadenitis, disseminated disease, and skin and soft tissue infection. Disseminated disease can occur in severely immuno-compromised patients (Griffith et al., 2007).

Among NTM species, *M. avium* attracts serious attention, as is the most common cause of non-tuberculous pulmonary disease worldwide. This species includes four subspecies: *M. avium avium* (MAA), *M. avium hominissuis* (MAH), *M. avium paratuberculosis* (MAP) and *M. avium silvaticum* (MAS) (Thorel et al., 1990; Turenne et al., 2008). MAA and MAS are specific avian pathogens causing a tuberculosis-like disease in birds (Dvorska et al., 2003).

MAP is a well-known pathogen causing Johne's disease (Harris and Barletta, 2001) and may have a role in human Crohne's disease (Feller et al., 2007). Of importance, MAH causes pulmonary infections in humans in which the normal T cell immunity is compromised.

1.2 Pathogenesis

Virulent mycobacteria reside in the phagosomes in infected macrophages and prevent killing by blocking the phago-lysosome fusion (Danelishvili et al., 2007; Rocco and Irani, 2011; Russell, 2013). This ability of virulent mycobacteria to survive and multiply in macrophages is considered as the key for their pathogenicity, latency and transmission. All virulent mycobacteria like *Mtb*, *M. bovis, M. avium* and *M. marinum* dwell in macrophages by modifying the phagosome, the most hostile environment, according to their requirements, which is the signature evasion mechanism of virulent mycobacteria (Danelishvili et al., 2007; Early and Bermudez, 2011; Rocco and Irani, 2011; Russell, 2013).

Despite the differences between *M. avium* and *Mtb*, the basic pathogenic behavior in promoting their survival in infected macrophages, the primary host cell is similar. *M. avium* invades the human host via either the respiratory tract or the intestinal epithelium: the latter route of infection rather causes rapid dissemination than the former. *M. avium* interacts with various receptors to gain access into the macrophages. Macrophages express an array of pathogen recognition receptors (PRRs) and phagocytic receptors that play a crucial role in their recognition and response to pathogens. For example complement receptors (except, CR3 and CR4) (Bermudez et al., 1999; Bohlson et al., 2001), mannose receptors, fibronectin (Bermudez et al., 1991; Polotsky et al., 1997), and type A scavenger receptors are involved in this process. Also toll-like receptors (TLRs) 2 and 9 are important for *M. avium* recognition (Appelberg, 2006; Feng et al., 2003; Gomes et al., 2004; Gomes et al., 2008). This recognition leads to the phagocytosis of pathogens and the resulting combinatorial signals from these receptors are important for the activation of macrophages and thereby, are essential for the initiation of the innate immune response (Akira et al., 2006; Schafer et al., 2009; Takeuchi and Akira, 2010). Once, they are phagocytized by macrophages, they reside in a plasma membrane derived intracellular vesicle called phagosomes. Microorganisms in

the phagosomes are killed and digested upon fusion with lysosomes in their maturation process, forming phago-lysosomes (Desjardins et al., 1994; Vieira et al., 2002). Similar to other pathogenic mycobacteria, *M. avium* inhibits phagosome maturation, by preventing acidification and by blocking fusion of phagosomes with extremely acidic lysosomes (Danelishvili et al., 2007; de Chastellier and Thilo, 2002).

Efficient killing of pathogens in macrophages requires distinct pro-inflammatory cytokines. Once the macrophage engulfs *M. avium*, it produces and secretes numerous chemokines, cytokines and their ligands, e.g. TNF- α, LT-a/b; IL-1, -6, -12, -18; G-CSF and GM-CSF; CXCL-1, -2, -3, -8; CCL-2, -3, -4, -5, -20 (Blumenthal et al., 2005; Sarmento and Appelberg, 1996; Shiratsuchi and Ellner, 2001) which induce the development of pro-inflammatory responses. It has been shown that infection of macrophages with various mycobacteria strain types induce a differential pattern of cytokines in vitro (Chacon-Salinas et al., 2005).

It is evident that activation of TLR 2/MAPK leads to activation of immune response in macrophages. Subsequent activation and nuclear translocation of NF-kB occurs, which greatly enhances mycobacterial killing by production and secretion of pro-inflammatory cytokines, and facilitating phago-lysosome fusion (Gutierrez et al., 2008; Pathak et al., 2004). Interference with the activation of toll-like receptors (TLRs), in activation of host antimicrobial responses possibly by surface molecules may promote their survival in the macrophages (Early and Bermudez, 2011). A substantial amount of evidence indicated that cell wall glyco-peptidolipids (GPLs) are responsible for pathogenesis and specific GPLs are associated with virulence (Bhatnagar and Schorey, 2006). The protective responses against *M. avium* largely depend upon activation of macrophage oxidative/bactericidal functions by type 1 pro-inflammatory cytokines provided by immune T lymphocytes and other cell types (Ehlers et al., 2000; Florido et al., 2005; Petrofsky and Bermudez, 2005). *In vitro* studies and animal infection models have shown that phylogenetically diverse mycobacteria strains exhibit markedly different virulence and pathogenesis phenotypes in macrophages (Early and Bermudez, 2011; Lopez et al., 2003; Manabe et al., 2003; Manca et al., 2004; Manca et al., 2001; Meyer et al., 1998; von Reyn et al., 1995).

Though, *M. avium* is not considered as a public health problem, it is increasingly recognized as a relevant pathogen due to its overall increasing incidence not only in patients with immunosuppression, but also in immuno-competent individuals and its high case fatality (Henry et al., 2004; Marras and Daley, 2002; McGrath et al., 2008). Studying *M. avium* biology and pathogenesis is important because its clinical appearance and relevance (Chatterjee and Khoo, 2001; Rocco and Irani, 2011) is supposedly interlinked with the mechanisms by which some species of the genus are pathogenic while others are not (Behr, 2008; Grubek-Jaworska et al., 2009). The *in vitro* virulence of *M. avium* isolates obtained from various sources is characterized by determining their ability to multiply in primary human monocyte derived macrophages. We have previously systematically characterized *M. avium* human clinical isolates and environmental isolates based on their ability to replicate in human macrophages. Along this study we found that the macrophage-induced gene (*mig*) is quite consistently expressed when the bacilli are growing within macrophages making it a potential marker for clinical pathogenicity and *in vitro* virulence of *M. avium* isolates (Meyer et al., 1998; Plum and Clark-Curtiss, 1994). Analyses of growth rate of these isolates in macrophages suggested that the *in vitro* virulence of these isolates vary at length between and among clinical and environmental isolates. From the panel of those previously characterized strains we have chosen SCH 228 as a strain that exhibited a fast growth rate in macrophages (≈ 18 h) and SCH 215 which was only slowly replicating in macrophages in order to characterize their interaction with neutrophils.

1.3 Mycobacteria and neutrophils

Neutrophils are typically the first responders in host defense against invading pathogens. Neutrophils destroy pathogens by both oxidative and non-oxidative mechanisms. They are recruited to the site of mycobacterial infection and are the most predominantly infected phagocytic cells in human tuberculosis (Eum et al., 2010). However, the role of neutrophils in mycobacterial infection is not fully understood. There is clinical evidence based on the beneficial impact of the application of G-CSF on survival of AIDS patients suffering from *M. avium* infection (Keiser et al., 1998). Martineau et al. (2007) showed that neutrophils

contribute significantly to the control of *Mtb* in human blood. Some earlier murine studies reported a protective role for neutrophils in early infection with *Mtb* as well as with *M. avium* (Barrios-Payan et al., 2006; Pedrosa et al., 2000; Sugawara et al., 2004). On the contrary, a study showed that neutrophils do not contribute to the control of *Mtb* (Seiler et al., 2000). Furthermore, a neutrophil-driven, interferon (IFN)-inducible transcript signature in human whole blood was recently identified that correlated with clinical severity of TB, raising the possibility that neutrophils may directly contribute to disease pathogenesis (Berry et al., 2010). Similar to macrophages, neutrophils have been shown of being capable to phagocytize and kill at least some mycobacterial species. However, a systematic analysis of the cell-autonomous immune regulation of human neutrophils upon infection with human clinical *M. avium hominissuis* isolates was not undertaken so far. Moreover, it is widely accepted that only a better understanding of the pathogenic processes associated with *M. avium* infection and disease will lead to the development of effective tools to control these conditionally pathogenic bacteria.

1.4 Inhibition of phago-lysosomal fusion

Macrophages express an array of pathogen recognition receptors (PRRs) and phagocytic receptors that play a crucial role in their recognition and response to pathogens. Many studies have shown that receptors such as Toll-like receptors (TLR-2, 4, 6 and 9) (Heldwein and Fenton, 2002; Jo et al., 2007; Means et al., 1999; Quesniaux et al., 2004) mannose receptors (McGreal et al., 2005), DC-SIGN, Dectin-1, and Mincle (Torrelles et al., 2006), the complement C3 receptors, Fcγ phagocytic receptors, CD-14 and NOD-like receptors (NLRs) are involved in the recognition process. This recognition leads to the phagocytosis of pathogens and the resulting combinatorial signals from these receptors are important for the activation of macrophages and thereby, are essential for the initiation of the innate immune response (Akira et al., 2006; Schafer et al., 2009; Takeuchi and Akira, 2010).

Phagocytosis of invading bacteria by professional phagocytes such as macrophages, DCs and neutrophils results in the formation of a plasma membrane derived intracellular vesicle around the pathogen. This newly formed cellular compartment is called phagosome in which pathogenic microorganisms are killed and digested upon fusion with lysosomes in their

maturation process, forming phago-lysosomes. After detachment from the host cell membrane, a nascent phagosome undergoes a series of progressive maturation processes involving fission and fusion events that modify the composition of the membrane. This process highly depends on the interaction of the phagosome with the endocytic pathway; phagosomes sequentially fuse with early (sorting) endosomes, late endosomes, and eventually with lysosomes (Desjardins et al., 1994; Vieira et al., 2002). This process enables nascent phagosomes to gradually acquire the properties of the donor organelles, including the distinct membrane markers and the increasingly acidic pH value. As a consequence, a phagosome ultimately matures into a hybrid intracellular compartment called phago-lysosomes. Phago-lysosomes contain various digestive enzymes, including proteases, nucleases, lipases, and β-galactosidases, which display optimal activities in the highly acidic condition (pH ≤5.0) and efficiently degrade luminal contents (Vieira et al., 2002)

However, during *Mtb* infection, the phagosome maturation pathway is altered via multiple mechanisms to disrupt normal phagocyte effector functions (Deretic et al., 2006; Russell, 2001). The early maturation process of the *Mtb* phagosome includes fusion with early endosomes, wherein both Rab5 and transferrin receptors are found associated with it. However, the pathogen prevents recruitment of Rab5 effector proteins, such as early endosomal auto antigen 1 (EEA1) and hVPs35 (Fratti et al., 2001) that are required for further maturation of the phagosome. Absence of Rab5 effector proteins on *Mtb* phagosomes prevents docking and delivery of lysosomal hydrolases, cathepsins, and vacuolar ATPases. Ultimately, these defects result in a failure in conversion of a Rab5 (an early endosomal marker) containing phagosome to a Rab7 (a late endosomal marker) containing phagosome (Via et al., 1997).

1.5 Regulation of cytokines

Efficient killing of pathogens in macrophages require adequate and appropriate pro-inflammatory cytokines. Macrophages produce pro-inflammatory cytokines and chemokines in order to mount effective immune response against pathogens. Once the macrophage engulfs *Mtb*, it produces several cytokines such as IL-2, IFN-γ, IL-6, IL-1α/β, IL- 12, and

TNF-α (Kellar et al., 2011) and chemokines including CCL2, CCL3, CCL5, CCL7, CCL12, CXCL2, CXCL8, and CXCL10 (Algood et al., 2003), which induce the development of pro-inflammatory responses. Release of these chemokines is associated with activation of microbicidal responses promoting the migration of different cell subpopulations towards the *Mtb*-infected tissues to form granulomas (Serbina et al., 2008). Several studies have investigated the effects of chemokines in the function and recruitment of monocytes following the infection with *Mtb*. They promote monocytes, DCs, activated macrophages, polymorphonuclear cells (particularly neutrophils), and T lymphocytes migration to bronchoalveolar spaces during pulmonary TB (Gonzalez-Juarrero et al., 2003).

Mycobacteria are endowed with the unique capacities to modulate fundamental inflammatory processes, such as recruitment of immune cells to the infected lung and production of critical pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ). In addition, mycobacteria interfere with biochemical pathways involved in the production of eicosanoids and other lipid mediators that affects the fate of the infected cells. Many studies have shown that infection of macrophages with various *Mtb* strain types induce a differential pattern of cytokines *in vitro* (Chacon-Salinas et al., 2005). Recently, *in vitro* studies and animal infection models have shown that phylogenetically diverse *Mtb* strains exhibit markedly different virulence and pathogenesis phenotypes in macrophages (Lopez et al., 2003; Manabe et al., 2003; Manca et al., 2004; Manca et al., 2001).

1.6 ROS production

Production of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS) are one of the killing mechanisms in macrophages. The rapid generation of ROS is critical for host defense against bacteria and fungi. In addition to its ability to kill pathogens, ROS has broad signaling functions (Kumar et al., 2011). The NADPH oxidase family protein complexes generate ROS and are the main sources of ROS both in activated neutrophils and macrophages. The early events in *Mtb* infection involve the phagocytosis of the bacilli by alveolar macrophages and often their immediate killing by different macrophage bactericidal

mechanisms, including RNI and ROS (Zuniga et al., 2012).

1.7 Neutrophils

Polymorphonuclear neutrophils (PMNs) are the most abundant white blood cells in the human circulation. They are key components of the first line of defense against bacterial and fungal pathogens, and they also participate in the development of the inflammatory reaction (Nathan, 2006). For long time, it was thought that neutrophils are short lived and die soon after their antibacterial function. Discoveries during the 1990s and early 2000s made immunologists to appreciate the amazing complexity and sophistication of neutrophil functions. It became evident that neutrophils release various cytokines and chemokines and contribute to orchestrating the immune/inflammatory response (Cassatella, 1995). Highly sophisticated machinery directing neutrophil migration (Nourshargh et al., 2010) and a complexity of neutrophil granules (Borregaard et al., 2007) also came to be known. In addition, the concept that release of granular contents (Reeves et al., 2002) and formation of neutrophil extracellular traps (NETs) (Brinkmann et al., 2004) are involved in the killing mechanisms of neutrophils underscored the fact that neutrophils use complex mechanisms to perform their immune defensive functions (Amulic et al., 2012; Mantovani et al., 2011; Nathan, 2006). In the past few years, scientists have witnessed the novel roles of neutrophils in immunity against intracellular pathogens such as viruses and intracellular bacteria; shaping of adaptive immunity at different levels; and roles in diseases such as allergy and anaphylaxis, metabolic diseases, atherosclerosis, or thrombus formation. Approaches like antibody (anti-Gr1 or anti-Ly6G)-mediated depletion of neutrophils in mice (Daley et al., 2008) or genetic manipulations leading to the partial or complete genetic deletion of the neutrophil lineage (Hock et al., 2003; Jonsson et al., 2005; Ordonez-Rueda et al., 2012) helped to understand the role of neutrophils in immunity.

1.8 Neutrophil reservoirs

Under physiological conditions, nearly 50% of the circulating neutrophils are mature pool of

granulocytes. These neutrophils can be found in the bone marrow, spleen, liver and lung (Summers et al., 2010). The lung seems to be enriched in mature neutrophils than other organs (Kreisel et al., 2010; Sibille and Marchandise, 1993). However, it is not clearly understood how and why neutrophils are concentrated within these organs. One explanation could be that these organs are reservoirs of mature neutrophils, which can be rapidly deployed to sites of inflammation or infection. However, it is also possible that neutrophils are constantly patrolling in these organs in search of tissue damage or microbial invasion.

1.9 Maturation of neutrophils

Neutrophils are continuously generated in the bone marrow from myeloid precursors, a process called terminal granulocytopoiesis. Their daily production can reach up to 2×10^{11} cells (Borregaard, 2010). Interleukin-17A (IL-17A) synthesized by T cells induces granulocyte colony stimulating factor (G-CSF), which in turn controls this maturation process of neutrophils (Ley et al., 2006). Release of IL-17A is in turn under the control of IL-23 originating from tissue-resident macrophages and dendritic cells. During inflammation the number of neutrophils that infiltrate in tissues increases, and these pathogen-induced apoptotic cells are removed by macrophages and dendritic cells. This neutrophil clearance process results in down regulation of IL-23 synthesis by those cells and thus reduces G-CSF release (Stark et al., 2005). During maturation, the neutrophil goes through several stages, namely myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell and, finally, polymorphonuclear (segmented) cell. The different subsets of granules such as azurophilic, specific granules, and gelatinase granules and secretory vesicles are formed sequentially during maturation from promyelocytes (Borregaard, 2010).

1.10 Neutrophil migration

Neutrophils are the first responders to inflammatory stimuli such as bacterial infection or tissue injury and this requires migration from the circulation to the site of inflammation. In

the resting state, neutrophils are poorly adherent and exist in a spherical shape. A rapid change in cell morphology occurs in response to inflammatory stimuli such as formyl-Met-Leu-Phe (fMLP), IL-8 or bacterial products and become polarized and migrate towards the inflammatory mediators. Migration of neutrophils involves many well-coordinated interactions with vascular endothelium. This can be divided into four steps; capture, rolling, arrest and trans-endothelial migration (TEM).

Initiation of migration begins with the "capture" of PMNs by the vessel wall from flowing blood followed by their "rolling" along the vessel wall. This process of margination is a normal behavior of circulating PMNs. However, when appropriate stimuli are sensed, rolling of neutrophil comes to a halt and neutrophils become firmly attached to ECs. Both the process of capture and rolling is due to the reversible binding of transmembrane glycoprotein adhesive molecules called selectins, which are found on both PMNs and ECs. L-selectin expressed on the surface of neutrophils, allows loose tethering for the latter to roll along the endothelium. that later induces conformational changes in integrin adhesion molecules expressed on endothelial cells which results in strong adherence and leading to rolling arrest. Rolling arrest can also be mediated by binding of chemoattractants such as IL-8 to neutrophil receptors.

It has been postulated that TEM is initiated by either a receptor- mediated event in response to an inflammatory cytokine or an event propagated from signals from activated selectins. Down-regulation of selectins induces expression of integrins such as platelet-endothelial cell adhesion molecule-1 (PECAM-1) in a timely and sequential manner, which finally leads to the successful migration (Woodfin et al., 2009). Migration of neutrophils through endothelial cells to an inflamed site is guided by a chemotactic gradient. For example the exposure of neutrophils to chemoattractants such as fMLP and complement component 5a (C5a) induces cellular polarization of chemoreceptors and formation of actin-rich pseudopodia at the leading edge of the cell (Servant et al., 2000). Adhesion and migration of neutrophils are accompanied by release of proteases and receptors upon degranulation (Hanlon et al., 1991). PMNs possess proteases capable of digesting collagen, laminin, and other extracellular components present in the vascular wall for the efficient migration towards the source of chemoattractants or the site of infection.

1.11 Activation of neutrophils

In the circulation of healthy adults, neutrophils exist in a resting state, which ensures that their toxic intracellular contents are not accidentally released to damage host tissue. Activation or priming of neutrophils occurs via two separate mechanisms. Resting neutrophils can become rapidly primed when intracellular granules possessing preformed receptors are mobilized to the plasma membrane. This rapid process enhances the number of plasma membrane receptors by mechanisms that do not require protein synthesis. However, often, neutrophils can become primed by agents that include bacterial products and cytokines or chemokines, e.g. TNF-α, GM-CSF, IL-8 and IFN-γ (Hallett and Lloyds, 1995) and primed neutrophils are then mobilized to the site of infection or inflammation, where they encounter activating signals to trigger bacterial killing.

1.12 Recognition of pathogens

It has long been known that *N*-formyl peptides induce neutrophil chemotaxis and functional activation via the seven-transmembrane G protein-coupled receptor FPR1. The production of formylated proteins is restricted to bacteria and mitochondria (Zhang et al., 2010), and therefore FPR1 fulfills the criteria of a pattern recognition receptor (PRR) recognizing microbial moieties and tissue damage. Neutrophils express a vast repertoire of PRRs including all members of the Toll-like receptor (TLR) family with the exception of TLR3 (Hayashi et al., 2003); the C-type lectin receptors dectin 1 (Greenblatt et al., 2010) and CLEC2 (not expressed in mouse neutrophils) (Kerrigan et al., 2009) and cytoplasmic sensors of ribonucleic acids (RIG-I and MDA5) (Tamassia et al., 2008). In addition, neutrophils express nucleotide-binding oligomerization domain protein 1 (NOD1) (Clarke et al., 2010), although the expression and function of the NOD-like receptors (NLRs) that are components of the inflammasome have not been carefully studied. The sensing of pathogens and tissue damage through these PRRs, together with lymphoid cell-derived signals, activates the effector functions of neutrophils. These include the production of reactive oxygen intermediates (ROI), lytic enzymes and antimicrobial peptides, and release of NETs (Segal, 2005).

1.13 Killing mechanisms

Neutrophils can eliminate both intra- and extracellular pathogens by multiple mechanisms. When neutrophils encounter microorganisms, they phagocytize, encapsulate them in phagsomes, and kill the pathogens using NADPH oxygenase-dependent mechanisms and/or antibacterial proteins released into the phagsomes from various granules (Borregaard, 2010; Hager et al., 2010). Soon after phagocytosis of the pathogen, contents of granules, especially specific and azurophilic granules fuse with the phagosomes. Upon further activation of neutrophils, accumulation of granular contents increases tremendously leading to the bulging of phagosomes. As a result of this accumulation and pumping in of various ions, the pH of the phagosome starts to decrease, which induces the fusion of lysosomes. Acidified condition of phagosomes is required for the phago-lysosomal fusion and for the optimal activity of various acid hydrolases and other contents of lysosomes in order to destroy pathogens. Neutrophils have developed a combinatorial mechanism, which involves granular contents and ROS to kill pathogens that have escaped from the oxidative mechanism of killing (Nordenfelt and Tapper, 2011; Winterbourn et al., 2006; Winterbourn and Kettle, 2013).

1.14 NADPH oxygenase-dependent killing mechanism

Oxygen radicals and their reaction products, collectively referred to as ROS, are produced as a consequence of NADPH oxidase activity, which pumps superoxides (O^-_2, H_2O_2 and $^{\cdot}OH^-$) into the phagocytic vacuole. The neutrophil NADPH oxidase system present mainly on the membrane of phagosomes and also on the plasma membrane is dormant in resting cells. When activated by soluble mediators (e.g., chemoattractant peptides and chemokines) and particulate stimuli (e.g., bacteria and immune complexes) it further interacts with cell-surface receptors to generate copious amount of ROS. Activation of phagocyte NADPH oxidase (NOX2) is mediated by the assembly of cytosolic subunits (p40^{phox}, p47^{phox}, and p67^{phox}) with the membrane subunits (p22^{phox} and gp91^{phox}). Phosphorylation mediated activation of p47^{phox} by protein kinases allows its membrane translocation and binding to p22^{phox}. On the other hand activation of Rac promotes its membrane translocation and its

binding to p67^{phox} , finally leading to the assembly of active NOX2 and superoxide anion production. Accumulation of superoxide can cause damage to the cells. However, cells have scavengers and anti-oxidant mechanisms to convert this superoxide into H_2O_2. Apart from ROS, hypochlorous acid (HOCl), that is a potent anti-microbial agent known for decades, is also produced in phagosomes. Myeloperoxidases of azurophilic granules fuse with the microbe-containing phagosomes containing ROS, where halides are also pumped in. These myeloperoxidases then catalyze the H_2O_2-dependent oxidation of halides forming HOCL.

1.15 Degranulation

The granule proteins of neutrophils are key effectors in the immune response and are released either into phagosomes or into the extracellular milieu, thus acting on either intra- or extra-cellular pathogens, respectively (Chertov et al., 2000). These proteins are essential for neutrophil-endothelial interaction, extravasation, phagocytosis, and elimination of microorganisms. Neutrophils contain four different granule subsets that are mobilized by stimulation and fuse with the cell or phagosomal membrane, resulting in exocytosis and/or exposure of soluble (present in their lumen) and membrane bound proteins. These granules are: azurophilic (also known as primary), specific (also known as secondary), gelatinase (also known as tertiary) and secretory granules (Faurschou and Borregaard, 2003). These classes of granules are formed sequentially during different stages of neutrophil differentiation in the bone marrow. The granules are classified according to their protein content and their differential ability to be exocytosed after neutrophil activation by inflammatory stimuli or phagocytosis of invading microorganisms. For example, secretory granules are more readily exocytosed than gelatinase granules, which in turn are more easily exocytosed than specific granules. Azurophilic granules only undergo limited exocytosis and are traditionally believed to contribute mainly to the intracellular degradation of microorganisms in the phago-lysosomes. These granules can be distinguished by their granule specific markers. Azurophilic granules contain high amounts of lysosomal enzymes (Faurschou and Borregaard, 2003) and are characterized by the presence of granulophysin (CD63) in their membranes (Cham et al., 1994). Their contents include myeloperoxidase

(MPO), bactericidal permeability-increasing protein, defensins, cathepsin G (a family of serine proteases) neutrophil elastase and proteinase 3. About one third of the total lysozyme is found in these granules (Baggiolini et al., 1969).

Peroxidase-negative granules can be divided into specific and gelatinase granules (Kjeldsen et al., 1992b). Gelatinase granules are the main reservoir of tissue-degrading enzymes, such as metalloproteinase and membrane receptors needed during PMN extravasation and diapedesis (Mollinedo et al., 1997). In contrast, specific granules participate mainly in the antimicrobial activities of the PMNs by mobilization of their arsenal of antimicrobial substances (*e.g.* alkaline phosphatases, collagenases, lactoferrin, NGAL, lysozyme, and hCAP18, the proform of LL-37) either to the phagosome or the exterior of the cell (Kjeldsen et al., 1992a; Mollinedo et al., 1997; Sengelov et al., 1995). These specific granules also contain unsaturated lactoferrin (that binds and sequesters iron and copper), neutrophil gelatinase-associated lipocalin and a number of membrane proteins including flavocytochrome b_{558} of the NADPH oxidase (Segal and Jones, 1979). The membrane of the gelatinase and specific granules contain markers such as CD66, CD15, or CD67. Secretory granules or vesicles are of endocytic origin that constitutes the main reservoir of a pool of membrane-associated receptors. Receptors stored in these vesicles are essential for neutrophil activation/function and are released easily in response to inflammatory molecules. These receptors are incorporated into the plasma membrane after release of the vesicles (Sengelov et al., 1994). For example, receptors such as β2-integrins, CR1, formyl peptide receptors (fpr), CD14, CD16 and the metalloproteinase leukolysin are displayed on the surface of neutrophils. This type of vesicle can be identified by the presence of their marker, CD35.

1.16 NETs

Neutrophils have developed specialized mechanisms to fight pathogens both in static and under in vivo flow conditions. Neutrophils have been shown to release their DNA, forming a net-like trap called neutrophil extracellular traps (NETs) that trap various bacteria

(Brinkmann et al., 2004). The process of formation of NETs is called NETosis. NETs are composed of nuclear components (such as DNA and histones) and are decorated by proteins from primary granules (such as myeloperoxidase and neutrophil elastase), secondary granules (such as lactoferrin and pentraxin 3 (PTX3)) and tertiary granules (such as matrix metalloproteinase 9 (MMP9) and peptidoglycan recognition protein short (PGRP-S). NET-localized molecules have a diverse repertoire of functions, including microbial recognition, antimicrobial activity and tissue remodeling. Also, NETs were found to have pro-inflammatory role (Gupta et al., 2010).

1.17 Production of inflammatory mediators

In response to a variety of inflammatory stimuli and pathological situations, primed neutrophils synthesize and secrete a panel of cytokines, chemokines, leukotrienes and prostaglandins. These secreted cytokines not only activate themselves but also activate other cells of the immune system. Activated neutrophils have been reported to secrete cytokines (interleukin (IL)-1β, IL-1RA, IL-4, IL-6, IL-10, IL-12, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ)) and chemokines (IL-8 and interferon inducible protein 10 (IP-10)) (Cassatella, 1999). The secretion of various cytokines and chemokines by activated PMNs is regulated by immunoregulatory cytokines such as interferon gamma (IFN-γ), IL-4 and IL-10. Neutrophils also secrete leukotriene B4 (LTB4) and prostaglandin E2 (PGE2), which are synthesized from arachidonic acid by lipoxygenases and cyclo-oxygenases, respectively.

1.18 Neutrophil and macrophage interaction

Being a fast responder, neutrophils are instrumental for both innate and adaptive immunity. After reaching the site of infection, they get activated by host and bacterial stimuli and release their mediators to attract and activate other innate immune cells like, macrophages, monocytes, epithelial cells, mast cells, endothelial cells and platelets, etc. In spite of their similar capacities such as phagocytosis of invading pathogens and immuno-modulatory

properties, both macrophages and neutrophils work as partners complementing and cooperating with each other (Silva, 2010b). It is thought that neutrophils that arrive to the site of infection first release macrophage inflammatory protein-1α (MIP-1α) and MIP-1β to attract monocytes and macrophages (Bennouna et al., 2003). In addition, release of (IFN)-γ from activated neutrophils causes activation of macrophages.

Macrophages, by releasing TNF-α, IL- 1β, G-CSF and GM-CSF at the site of infection increase survival of recruited neutrophils (Lee et al., 1993; Yamashiro et al., 2001). Recently, it has been reported that macrophages release exosomes to communicate with other immune cells. Exosomes are small vesicles formed within multi-vesicular bodies (MVB) and are released outside the cell upon fusion of MVB with plasma membrane (Chaput and Thery, 2011; Robbins and Morelli, 2014; Schorey and Bhatnagar, 2008; Thery et al., 2001; Thery et al., 2002). Also, it has been proposed that exosomes that are released from the infected macrophages may have different roles depending upon the contents they carry (Robbins and Morelli, 2014).

By virtue of their cell mediators, it is possible for activated neutrophils to influence macrophage differentiation into pro-inflammatory or anti-inflammatory subtypes (Chertov et al., 1997; Tsuda et al., 2004). Granular proteins especially myeloperoxidases from the neutrophils leads to the induction of reactive oxygen species (ROS) along with other pro-inflammatory cytokines (i.e. TNF-α, IL-1, IL-6, IL-8 and GM-CSF) from macrophages (Ethuin et al., 2004; Lefkowitz et al., 2000). The interaction in the form of congregation of neutrophils and macrophages has been reported in many infectious diseases like TB, listeriosis, salmonellosis and legionellosis (Serbina et al., 2008; Tam et al., 2008; Torrado et al., 2007). During such close interaction, neutrophils have been shown to be able to increase the antimicrobial action of macrophages by providing potent antimicrobial molecules for which macrophages are deficient (Miyakawa et al., 1996). In addition, apoptotic neutrophils have been shown to induce pro-inflammatory response in macrophages (Tan et al., 2006).

1.19 Objectives of the study

Neutrophils have been implicated in the innate immune response during the initial phase of mycobacterial infection. However, the role of neutrophils in mycobacterial infection is not fully understood. While the mechanisms by which virulent mycobacteria are able to propagate inside macrophages by interfering with the phagosomal maturation process are known, whether virulent mycobacteria impact on defense mechanisms of neutrophils still remains largely unknown. Also, the effect of interaction between neutrophils and infected macrophages on intra-macrophage bacterial survival is not known. It has been reported that human clinical isolates, *M. avium hominissuis* SCH 228 and SCH 215 show differential *in vitro* virulence in human macrophages in terms of their generation time. However, such virulence-based behavior of these clinical isolates has not yet been studied in human neutrophils. Our group has previously shown that human neutrophils are able to phagocytize and kill the avian type strain *Mycobacterium avium* TMC 724 (*M. avium*) *in vitro*. Therefore, objective of this study was to investigate the differential cell-autonomous immune regulation of neutrophils upon infection with the two human clinical *M. avium hominissuis* isolates, SCH 228 and SCH 215.

2 Results

2.1 Both clinical *Mycobacterium avium* isolates, SCH 228 and SCH 215, prevent killing by human neutrophils by inhibiting phago-lysosome fusion

We have previously shown that human neutrophils phagocytize and kill the avian type strain, *Mycobacterium avium* TMC 724 *in vitro* (Hartmann et al., 2001). Here, we assessed the ability of human neutrophils to kill clinical isolates of *M. avium hominissuis* (SCH 228 and SCH 215) that were isolated from human patients. SCH 228 isolated from bone marrow of an AIDS patient and SCH 215 derived from the cervical lymph node of a 5 year old boy suffering from lymphadenitis colli exhibit differential growth rates in the HMDM model (Meyer et al., 1998). Freshly isolated neutrophils from healthy donors were allowed to phagocytize mycobacteria at a ratio of 1:1 (PMN/bacteria) for 15 min. Extracellular bacteria were then removed by differential sedimentation centrifugation. *M. avium* rescued from neutrophils at defined time points of the incubation period at 37°C, were cultured for 7–14 days and the colony forming units/1000 µl (CFUs/1000 µl) of *M. avium* were determined. Analysis of CFU showed that SCH 228 and SCH 215 had been phagocytized with similar efficiency at 15 min (Fig. 1). However, following phagocytosis CFUs did not decrease over time, indicating that human neutrophils could not kill these clinical *M. avium* isolates.

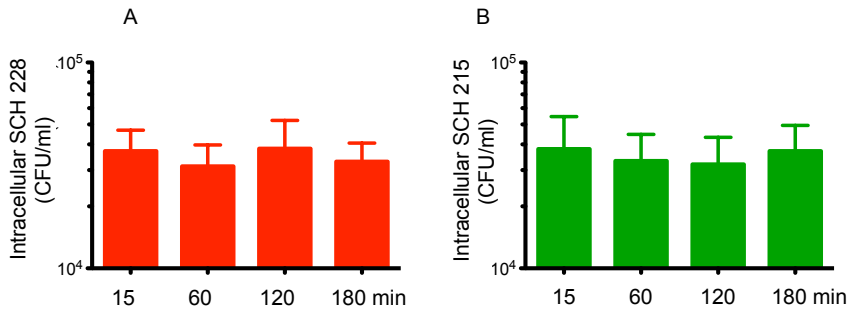


Figure 1 *M. avium* strains SCH 228 and SCH 215 are not killed by human neutrophils.

Neutrophils were infected with SCH 228 (**A**) or SCH 215 (**B**) at a ratio of 1:1 at 37°C and allowed to phagocytize for 15 min. After removal of extracellular bacteria, CFUs of intracellular bacteria were determined at the indicated time points (min). The graph shows the mean values of three independent experiments with duplicates at each time point in each experiment. Error bars indicate standard error of mean (SEM) (p >0.05, ns).

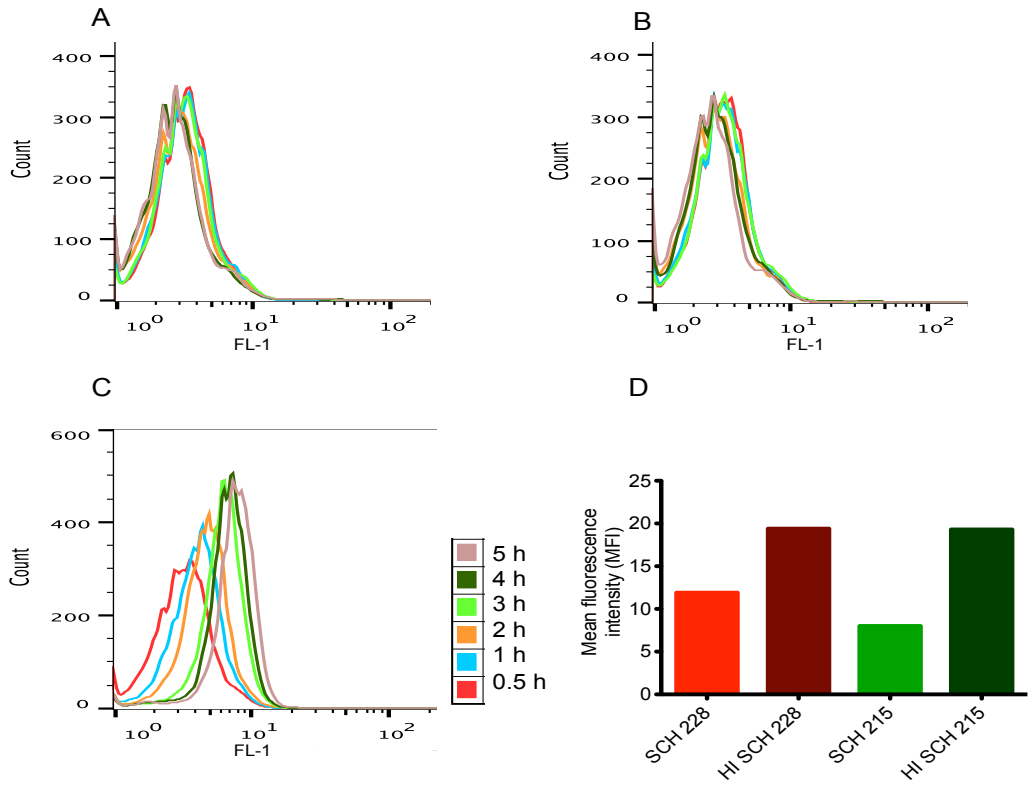


Figure 2 SCH 228 and SCH 215 *M. avium* inhibit phago-lysosomal processing in human neutrophils.

Human neutrophils were infected with C12-FDG-coated SCH 228 (**A**) or SCH 215 (**B**) or heat-inactivated mycobacteria (**D**) for 15 min at 37°C (ratio 1:20). Neutrophils fed with C12-FDG-coated magnetic beads (**C**) were used as positive control for this assay. After removal of extracellular mycobacteria, cells were incubated and fluorescence was analyzed by flow cytometry at the indicated time points (color scheme). Phagosomal processing is shown by the shift in the fluorescence over time. Processing of C12-FDG-coated heat-inactivated mycobacteria (HI) vs intact mycobacteria was analyzed after 2 h post-infection and represented as the mean fluorescence intensity (**D**). Results are representative of three independent experiments.

Virulent mycobacteria are known to reside within the phagosome of infected macrophages by inhibiting phago-lysosomal fusion thereby preventing their degradation (Rohde et al., 2007a; Rohde et al., 2007b; Russell, 2001; Russell et al., 2010b; Sweet et al., 2010). We therefore, asked whether inhibition of phago-lysosomal fusion in SCH 228 and SCH 215-infected human neutrophils could be responsible for the observed bacterial survival (CFU/ml). For this purpose, we employed C12 FDG (5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside), a substrate for β-galatosidase enzyme, present in the lysosome, to study phagosomal processing using FACS (Robinson et al., 2008). Mycobacteria coated with C12-FDG were used to infect neutrophils and chased for up to 5 h. Magnetic beads coated with C12- FDG served as a positive control in this assay. As shown in Fig. 2C, neutrophils fed with C12-FDG coated magnetic beads showed an increase in fluorescence over time suggesting that the galactopyranoside coating on magnetic beads is cleaved by lysosome resident β-galatosidase upon phago-lysosome fusion. On the contrary, neutrophils infected with C12-FDG coated SCH 228 or SCH 215 *M. avium* did not show such increase in fluorescence indicating that lysosomes did not fuse with the mycobacteria containing phagosomes (Fig. 2A and B). In order to test whether these isolates actively inhibit phago-lysosomal fusion, we used heat-inactivated mycobacteria for this assay. Neutrophils infected with C12-FDG coated heat-inactivated mycobacteria showed increased fluorescence compared to live mycobacteria suggesting that heat inactivated mycobacteria are not able to inhibit phago-lysosomal fusion as compared to live mycobacteria (Fig. 2D). Together, these data show that SCH 228 and SCH 215 *M. avium* clinical isolates prevent killing in human neutrophils by actively inhibiting the fusion of lysosomes with the phagosome.

2.2 *M. avium* SCH 228 and SCH 215 dampen the activation of p38 MAPK in human neutrophils

Clearance of pathogens by neutrophils upon phagocytosis requires efficient degranulation, production of reactive oxygen species (ROS) and phagosomal processing, which is aided by the induction of appropriate cytokines (Sabroe et al., 2005; Sabroe et al., 2003; Segal, 2005; Winterbourn and Kettle, 2013; Yoshimura and Takahashi, 2007). Activation of p38 MAPK in neutrophils is important for various functions including degranulation, increased ROS,

cytokine induction, etc. (Lee et al., 1994; Mocsai et al., 2000; Peroval et al., 2013; Thomas and Schroder, 2013; Zu et al., 1996; Zu et al., 1998). Therefore, we analyzed whether these clinical isolates have any influence on the activation of p38 MAPK pathway, by western blotting. The activation of p38 MAPK in infected neutrophils is reduced in the early time points from 15 min till 60 min (Fig. 3). Notably, this reduction in the activation of the pathway is stronger in SCH 228 infected neutrophils than in SCH 215-infected cells. Thus both SCH 228 and SCH 215 *M. avium* clinical isolates differentially dampen the activation of p38 MAPK signaling pathway.

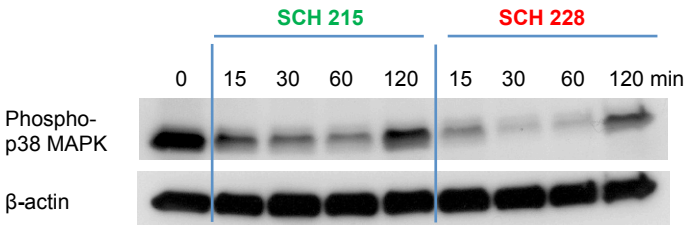


Figure 3 SCH 228 and SCH 215 dampen the phosphorylation and activation of p38 MAPK in human neutrophils.

Mycobacteria-infected human neutrophils were lysed at the indicated time points and phosphorylation of p38 MAPK was assessed by immuno-blotting using anti-phospho p38 MAPK antibody as described in Materials and Methods.

2.3 SCH 228 and SCH 215 *M. avium* dampen p38 MAPK mediated degranulation in human neutrophils

Killing of intracellular pathogens by neutrophils involves both oxidative and non-oxidative mechanisms. Degranulation, the non-oxidative mechanism is an important feature of neutrophils in the defense of both extra- and intracellular pathogens and is mediated by p38 MAPK activation (Borregaard, 2010; Borregaard et al., 2007; Faurschou and Borregaard, 2003; Prince et al., 2011; Segal, 2005; Thomas and Schroder, 2013; Winterbourn and Kettle, 2013). Expression and fusion of contents from various granules with phagosomes result in intra-phagosomal degradation of pathogens. Therefore, we investigated whether the observed interference of SCH 228 and SCH 215 *M. avium* with the phosphorylation and activation of p38 MAPK impacts on the degranulation process. For this purpose, human

neutrophils were infected with either SCH 228 or SCH 215 and cell surface expression of CD35, CD63, and CD66b was measured for assessing degranulation of secretory, azurophilic, and specific granules, respectively, by flow cytometry. The N-formylated peptide, fMLP, was used as a positive control for the p38 MAPK-mediated degranulation. Fig. 4 shows the cell surface expression of granule specific markers in human neutrophils at 15 min. fMLP induced cell surface expression of both CD66b and 35 (Fig. 4B and C; blue bar) but not CD63 (Fig. 4A, blue bar). In striking contrast, both isolates dampened the expression of CD66b and 35 markers in infected human neutrophils by ≈66 and 50%, respectively.

This inhibition in degranulation in *M. avium*-infected neutrophils was similar to the degranulation pattern in cells pre-incubated with p38 MAPK inhibitor (SB203580) for 30 min (brown bar) before being stimulated with fMLP indicating the role of p38 MAPK in degranulation. These data show that both SCH 228 and SCH 215 dampen p38 MAPK mediated degranulation in human neutrophils.

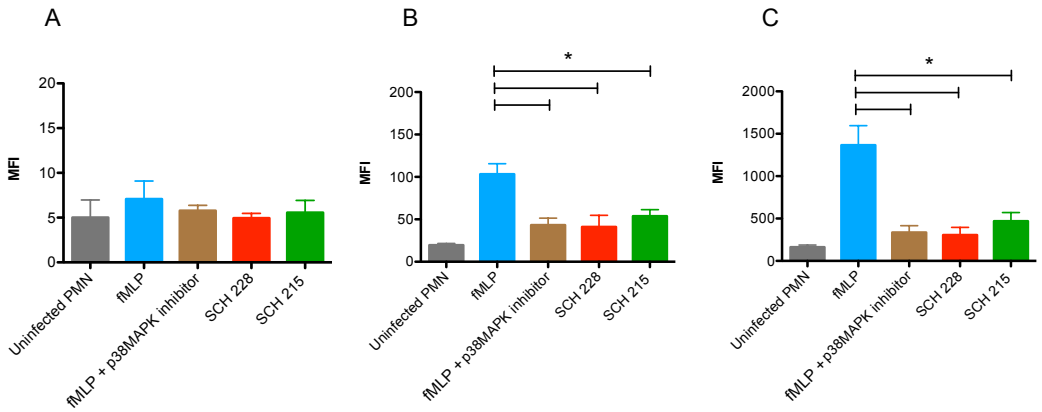


Figure 4 SCH 228 and SCH 215 *M. avium* dampen p38 MAPK mediated degranulation in neutrophils.

Neutrophils were infected with mycobacteria SCH 228 (red) or SCH 215 (green) at a ratio of 1:10 for 15min and infected cells were analyzed for the cell surface expression of granule specific markers CD63 (**A**), CD35 (**B**) and CD66b (**C**) for assessing degranulation of azurophilic, secretory and specific granules, respectively, by flow cytometry. Neutrophils were incubated in the presence of HBSS buffer (control; grey), fMLP (10 µM) for 15 min (blue) or p38 MAPK inhibitor (2 µM) for 30 min prior to stimulation with fMLP for 15 min (brown). Data represent mean ± SEM of three independent experiments (*p <0.05).

2.4 SCH 228 and SCH 215 *M. avium* induce ROS differentially in human neutrophils

Production and release of reactive oxygen species (ROS) is a crucial antibacterial mechanism of neutrophils, also mediated by p38 MAPK (Brumell et al., 1996; Makni-Maalej et al., 2013; Segal, 2005; Sue et al., 1997; Thomas and Schroder, 2013). ROS when released into the phagosome could by itself kill intra-phagosomal pathogens (Segal, 2005; Winterbourn and Kettle, 2013). As shown above SCH 228 reduced the activation of p38 MAPK pathway stronger than SCH 215 (Fig. 3). Using a luminol-based assay, we measured the ROS induction in neutrophils infected with either SCH 228 or SCH 215 for a period of 4 h (Fig. 5). ROS produced by uninfected neutrophils was considered as the baseline and fMLP was used as positive control for this assay. The kinetics of the ROS production revealed that fMLP induced ROS, reached its peak at 15 min and fell to baseline at 30 min (Fig. 5A, blue line). Surprisingly, SCH 215 induced a robust production of ROS after 15 min and reached its peak between 90-120 min (Fig. 5A, green line). The total amount of the accumulated ROS started to fall thereafter. In contrast, SCH 228 induced significantly lower levels of ROS in neutrophils but with similar kinetics as that of SCH 215 (Fig. 5A, red line). Notably, even after reaching their respective peaks, the levels of ROS did not return to basal levels. Instead, both isolates induced ROS in moderate amounts in neutrophils through out the entire period of the kinetics.

In order to prove the inhibitory effect of SCH 228 we measured the ROS induction in neutrophils infected with SCH 215 alone or in the presence of SCH 228. As expected, ROS induced in neutrophils co-infected with both SCH 215 and SCH 228 isolates was significantly reduced when compared to the ROS induced by SCH 215 alone (Fig. 5C, compare green and orange lines). These data indicate that SCH 228 inhibits ROS induction in the neutrophils. The ROS induced by these isolates decreased significantly in neutrophils that had been pre-treated with SB203580 (p38 MAPK inhibitor) suggesting that indeed p38 MAPK mediates ROS induction in infected neutrophils (Fig. 5B). Together these data demonstrate that these isolates differentially impede ROS induction in neutrophils by reducing the activation of p38 MAPK and SCH 228 is capable of inhibiting ROS induction by SCH 215.

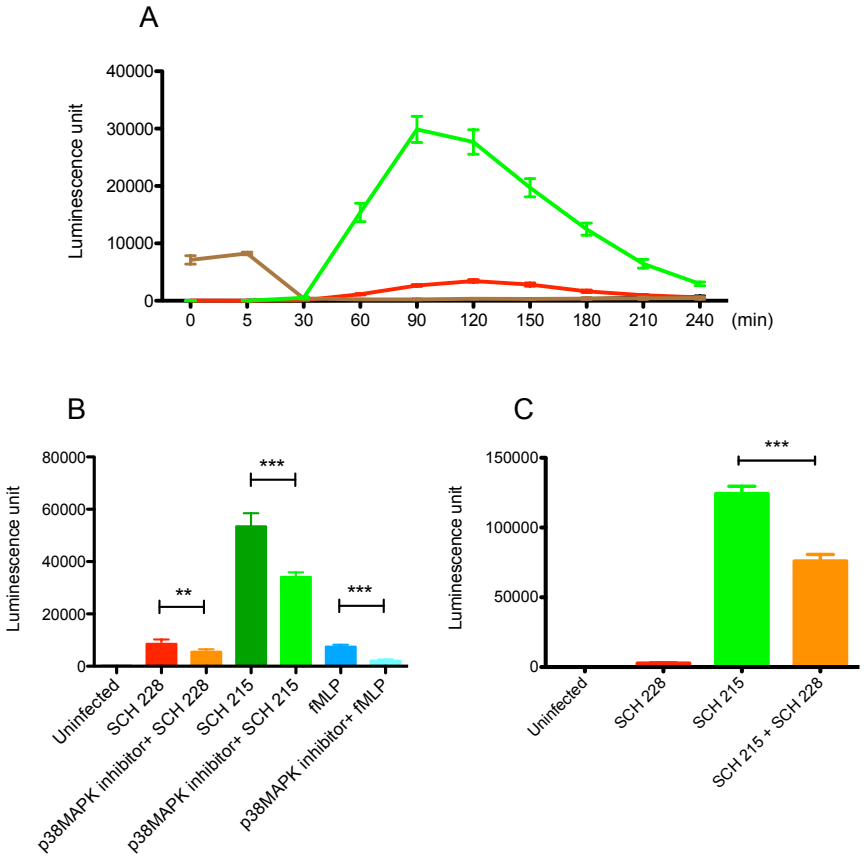


Figure 5 ROS induction in mycobacteria-infected human neutrophils.

A. Kinetics of the ROS induction in human neutrophils measured over a period of 4 h. Human neutrophils were incubated with TC medium, SCH 228 (red), SCH 215 (green) or fMLP (blue) (10 µM) and total ROS was measured using luminol-based assay. Result is representative of three independent experiments. **B.** p38 MAPK mediates the induction of ROS in mycobacteria-infected neutrophils. Neutrophils were pre-incubated with p38 MAPK inhibitor for 30 min before mycobacteria or fMLP was added. Data represent mean±SEM of the peak value for each sample from three different donors. **C.** SCH 228 inhibits ROS induced by SCH 215. Neutrophils were incubated with SCH 215 (Ratio 1:100) alone or with 228 (Ratio 1:50). Data represent mean±SEM of the peak value for each sample from three different donors (**p <0.005, ***p <0.0005).

2.5 SCH 228 and SCH 215 *M. avium* induce only certain cytokines differentially in infected neutrophils

Neutrophils release cytokines not only to support and maintain their own functions but also to communicate and influence the function of other immune cells (Jaillon et al., 2013; Makni-Maalej et al., 2013; Mantovani et al., 2011; Nathan, 2006; Silva, 2010b; Zu et al., 1996; Zu et al., 1998). Neutrophils have been shown to produce pro inflammatory cytokines such as IL-6, IL-8, IL-1β, TNF-α, and anti-inflammatory cytokines such as IL-1RA and IL-10, and induction of these cytokines is mediated by p38 MAPK (Chi et al., 2006; Herlaar and Brown, 1999; Lee et al., 1994; Mayer et al., 2013). Therefore, we investigated whether reduced activation of p38 MAPK pathways upon infection impacts on the cytokine release from human neutrophils. To this end, neutrophils were incubated with SCH 228 or SCH 215 and the concentration of various cytokines was measured in the supernatants after 6 h, using commercially available ELISA kits. Most of the cytokines including IL-6, IL-1β, TNF-α and IL-10 were not induced in the infected neutrophils as indicated by their concentration below the detection levels (data not shown). IL-1ra, the receptor antagonist of IL-1 was lower in the supernatant of neutrophils infected with SCH 228 than either SCH 215 infected or uninfected cells (Fig. 6B). On the other hand, infected neutrophils induced IL-8 production higher than uninfected neutrophils (Fig. 6A). SCH 215-infected neutrophils induced 8-fold increase in IL-8 production while SCH 228 induced about 3-fold (Fig. 6A, compare green and red lines). These cytokine data reveal that both isolates dampen cytokine production in neutrophils and differentially induce IL-8 production in human neutrophils.

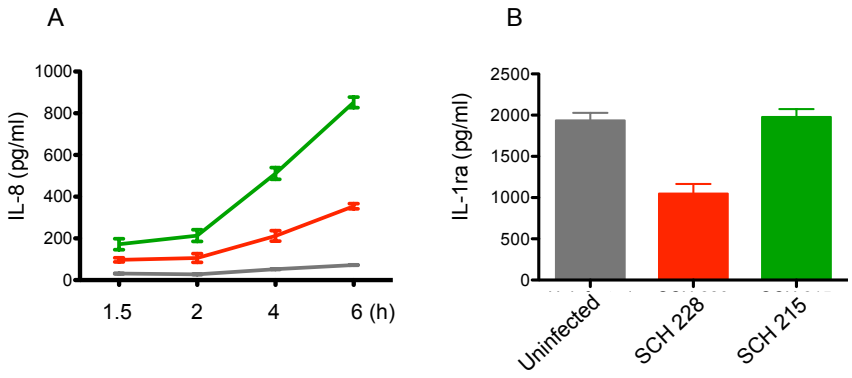


Figure 6 IL-8 and IL-1ra are differentially induced by SCH 228 and SCH 215 in infected neutrophils.

Neutrophils were incubated with SCH 228 (red) or SCH 215 (green) (ratio 1:50) and supernatants were collected at indicated time points (hours). Levels of various cytokines and chemokines were measured using commercially available kits as per manufacturer's instructions. **A.** Kinetics of IL-8 induction in infected neutrophils. **B.** IL-1ra levels in neutrophils after 6 h of incubation. Data represent mean±SEM from three different donors.

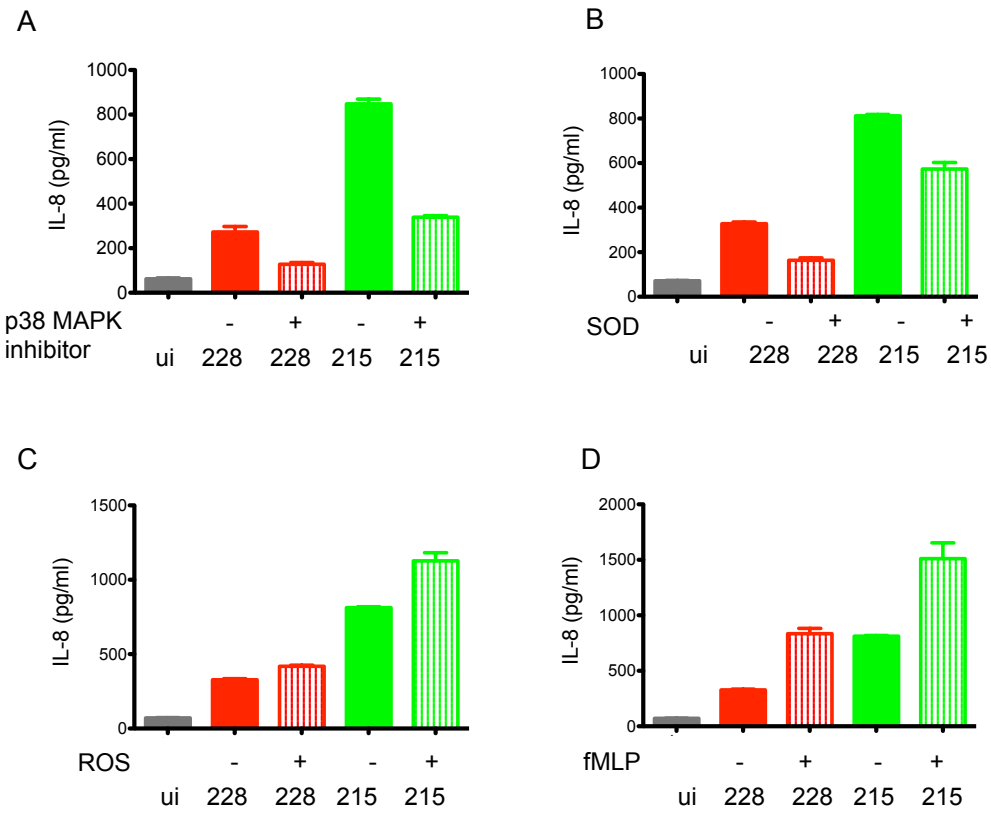


Figure 7 IL-8 induction in infected neutrophils is mediated by p38 MAPK via ROS.

Neutrophils pre-treated with p38 MAPK inhibitor (2 µM) (**A**) or fMLP (10 µM) (**D**) for 30 min or untreated neutrophils were incubated with mycobacteria alone or in presence of ROS (H2O2; 400 nM) (**C**) or SOD (50 µg/mL) (**B**). Supernatants were collected after 6 h and concentration of IL-8 was measured. Data represent mean±SEM from three different donors.

2.6 IL-8 induction in *M. avium* infected human neutrophils is p38 MAPK and ROS dependent

The kinetics of ROS production and IL-8 release show that ROS production happens earlier than IL-8 release. This observation leads to the hypothesis that P38 MAPK and ROS are important for the induction of IL-8. To test this hypothesis, first, we checked whether release of IL-8 is ROS- dependent. Inhibition of ROS using super oxide dismutase (SOD) a ROS-scavenger significantly reduced the levels of IL-8 (Fig. 7B). Moreover, exogenous addition of hydrogen peroxide (H_2O_2, ROS metabolite) to neutrophils enhanced IL-8 induction in neutrophils (Fig. 7C). These data confirm that the IL-8 induction in *M. avium*-infected human neutrophils is ROS-dependent. Next, we checked the involvement of p38 MAPK in IL-8 induction using p38 MAPK inhibitor and activator (fMLP). IL-8 induction in mycobacteria infected neutrophils that were pre-treated with SB203580 and fMLP was significantly reduced and induced, respectively (Fig. 7A and D). These data together indicate that the production of IL-8 in the infected neutrophils is mediated by p38 MAPK via ROS.

2.7 IL-8 induces degranulation in neutrophils

From our results, it is evident that the two clinical isolates of *M. avium hominissuis* prevent killing in neutrophils by dampening the activation of the p38 MAPK, a pathway involved in many neutrophil functions. As various processes of neutrophils cross talk and influence one another, it is tempting to find out whether the function of neutrophils such as ROS production, cytokine induction and degranulation are interlinked to one another. Our data showed that the IL-8 production is mediated by p38 MAPK and ROS. It is known that IL-8 primes neutrophils for ROS production. However, whether or not IL-8 has any influence on degranulation was not studied in neutrophils. To study this, neutrophils were stimulated with IL-8 for 15 min, and degranulation was assessed as mentioned above. Stimulation with IL-8 induced degranulation of all the vesicles in treated neutrophils compared to untreated neutrophils (Fig. 8). This result shows that IL-8 induction via ROS upon p38 MAPK activation induces the degranulation process.

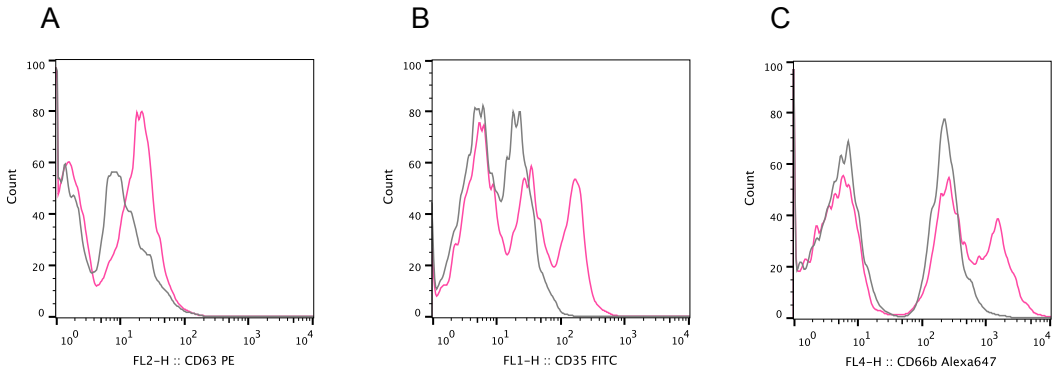


Figure 8 IL-8 induces degranulation in neutrophils.

Neutrophils were incubated with HBSS buffer (grey) or IL-8 (pink; 50 pg/mL) for 15 min and cells were analyzed for the cell surface expression of granule specific markers CD63 (**A**), CD35 (**B**) and CD66b (**C**) for assessing degranulation of azurophilic, secretory and specific granules, respectively, by flow cytometry. Results are representative of three independent experiments.

2.8 ROS and IL-8 do not affect the killing of SCH 228 by human neutrophils

SCH 228 dampens the activation of p38 MAPK pathway and thus, reduces ROS and IL-8 induction in neutrophils. IL-8 is a strong neutrophil priming agent. Given the fact that ROS mediated IL-8 induction and degranulation are interlinked (Fig. 7 and 8), we hypothesized that the addition of IL-8 or exogenous ROS could enhance bacterial clearance in infected neutrophils. To test this hypothesis, neutrophils pre-treated with IL-8 for 15 min or ROS for 30 min were infected with SCH 228 for 15 min and the CFU was compared to that of untreated SCH 228 infected cells. Neither IL-8 nor ROS treatment showed any effect on bacterial clearance (data not shown). These results suggest that exogenous addition of IL-8 or ROS does not enhance the killing of SCH 228 by neutrophils.

2.9 Stimulation by LPS does not affect killing of SCH 228 or SCH 215 by human neutrophils

Priming processes are critical in host defense against intracellular pathogens (Wright et al., 2010). Lipopolysaccharide (LPS) is also known as a strong priming agent that enhances many functions of neutrophils via TLR4 (Brinkmann et al., 2004). Hence, we asked if LPS -

stimulated neutrophils would exhibit a better clearance of bacteria than unstimulated ones. Neutrophils were stimulated with LPS for 30 min before they were infected with SCH 228 and SCH 215 and the number of bacteria present in the neutrophils was determined at selected time points. In contrast to our expectation, LPS-stimulated neutrophils did not show decreased CFUs compared to unstimulated neutrophils (Fig. 9). Thus, we conclude that both isolates SCH 215 and SCH 228, prevent killing by LPS-stimulated neutrophils.

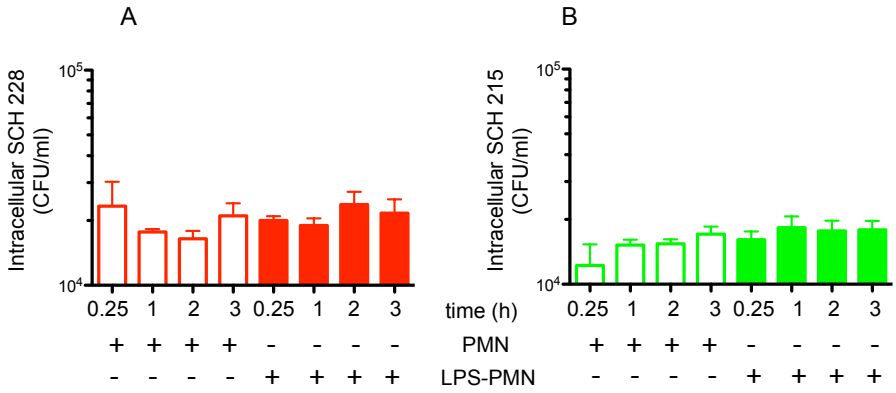


Figure 9 Priming with LPS does not enhance SCH 228 and SCH 215 killing by human neutrophils.

M. avium SCH 228 (**A**) and SCH 215 (**B**) were incubated with LPS stimulated neutrophils (filled bars) and unstimulated neutrophils (open bars) for indicated time points. Bacterial survival was determined by CFU counts of cell lysates. Data represent mean±SEM from three different donors. (p >0.05, ns).

2.10 *M. avium* isolates, SCH 228 and SCH 215, do not alter neutrophil death pathways

Microbial pathogens are capable of modulating host cell death as a virulence mechanism. Infected host cells are induced to undergo either apoptosis or necrosis through activation of various pathways including oxygen-dependent mechanisms (Abebe et al., 2011; Chen et al., 2006; Luo, 2011; Perskvist et al., 2000). Since, we have observed differential induction of ROS by these isolates in human neutrophils, we checked whether these clinical *M. avium* strains, SCH 228 and SCH 215 have any influence on the death of infected neutrophils. Using a commercially available Annexin V-apoptosis assay kit, we measured Annexin V positive and 7-AAD-postive cells among neutrophils that were infected with either SCH 228 or SCH 215 for 0 and 3 h. Uninfected neutrophils were considered as negative control while neutrophils incubated with phorbol myristate acetate (PMA) for 30 min were used as

positive control for this assay. Neutrophils stained positive for Annexin V alone represent apoptotic cells while cells positive for both Annexin V and 7-AAD were considered as late apoptotic or necrotic cells. At both time points, apoptosis of neutrophils infected with SCH 228 or SCH 215 was not significantly different than spontaneous apoptosis in uninfected neutrophils (Fig. 10A). On the other hand, PMA induced significant apoptotic cell death compared to uninfected neutrophils. Similarly, Annexin V^+ and 7-AAD^+ necrotic cell death of neutrophils incubated with these isolates was not different than uninfected neutrophils (Fig. 10B). These data suggest that virulence of SCH 228 or SCH 215 *M. avium* clinical isolates does not induce cell death in human neutrophils.

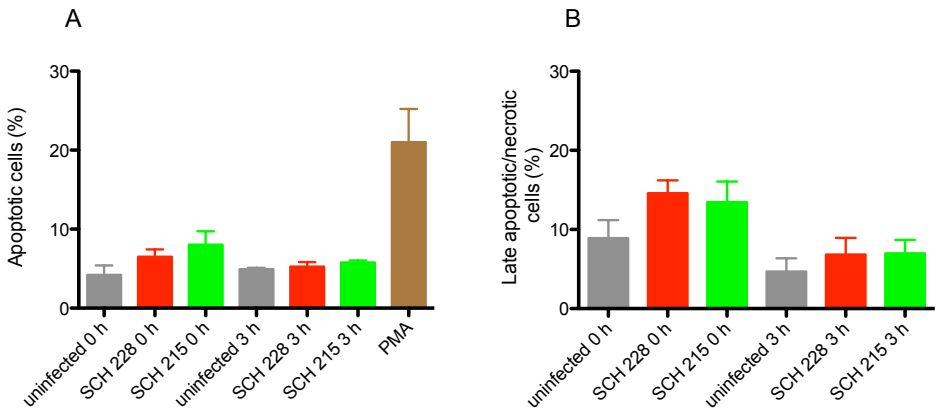


Figure 10 *M. avium* isolates, SCH 228 and SCH 215, do not alter neutrophil death pathways.

Neutrophils were infected with mycobacteria SCH 228 (red) or SCH 215 (green) at a ratio 1:10 at 37 °C for up to 3 h. At indicated times, cells were collected and dead cells were measured using commercially available Annexin V apoptosis kit, by flow cytometry. Uninfected neutrophils served as the negative control (grey) while cells incubated with PMA were considered as a positive control (brown). Cells stained positive for Annexin V represent apoptotic cells (A) while cells positive for both Annexin V and 7-AAD were considered as the late apoptotic/necrotic cells (B). Cell death measured after 15 min of phagocytosis was considered as 0 h. after Data represent mean±SEM from three different donors (p >0.05, ns).

2.11 Cytochalasin B/fMLP stimulated degranulation inhibits extracellular growth of SCH 228 and SCH 215 *M. avium*

Neutrophils discharge their storage granules both into phagosomes and to the extra cellular milieu (Borregaard et al., 2007; Faurschou and Borregaard, 2003; Prince et al., 2011; Segal,

2005; Thomas and Schroder, 2013). Neutrophils can be artificially induced to degranulate their granular contents into the supernatant. A combination of cytochalsin B (cytB), a fungal metabolite and fMLP has been shown to release contents from azurophilic and specific/gelatinase granules(Bentwood and Henson, 1980; Zarember et al., 2007). Soluble factors from the granules released outside the cell contribute to the extracellular killing of pathogens (Borregaard, 2010; Borregaard et al., 2007). Therefore, we asked whether stimulated degranulation from neutrophils could have an inhibitory effect on extracellular mycobacterial growth. First, cytB/fMLP induced degranulation after 30 min was assessed by the cell surface expression of granule specific markers by flow cytometry. As expected, incubation of neutrophils with cytB/fMLP resulted in increased expression of CD66b and CD63 compared to the solvent stimulated or unstimulated neutrophils (Fig. 11A and C). However, expression of CD35 in stimulated neutrophils was not increased (Fig. 11B). These data clearly demonstrate that cytB/fMLP stimulation in neutrophils induces degranulation of both azurophilic and specific/gelatinase granules. Having confirmed the degranulation from stimulated neutrophils, next, we asked if cytB/fMLP stimulated supernatant could inhibit extracellular growth of these isolates. Following cytB/fMLP stimulation, neutrophils were pelleted and 100 µl of supernatant from the equivalent of 10^7 neutrophils were added to 10^5 CFU of SCH 228 and SCH 215. The survival of these strains was determined by counting the bacteria in the supernatant after 24 and 48 h. Interestingly, CFU of both SCH 228 and SCH 215 was significantly decreased when incubated with supernatant from cytB/fMLP-stimulated neutrophils than that of solvent-stimulated or unstimulated neutrophils at 48 h (Fig. 11D and E). This result indicates that cytB/fMLP induced degranulation of neutrophils inhibits extracellular growth of these two clinical *M. avium* isolates.

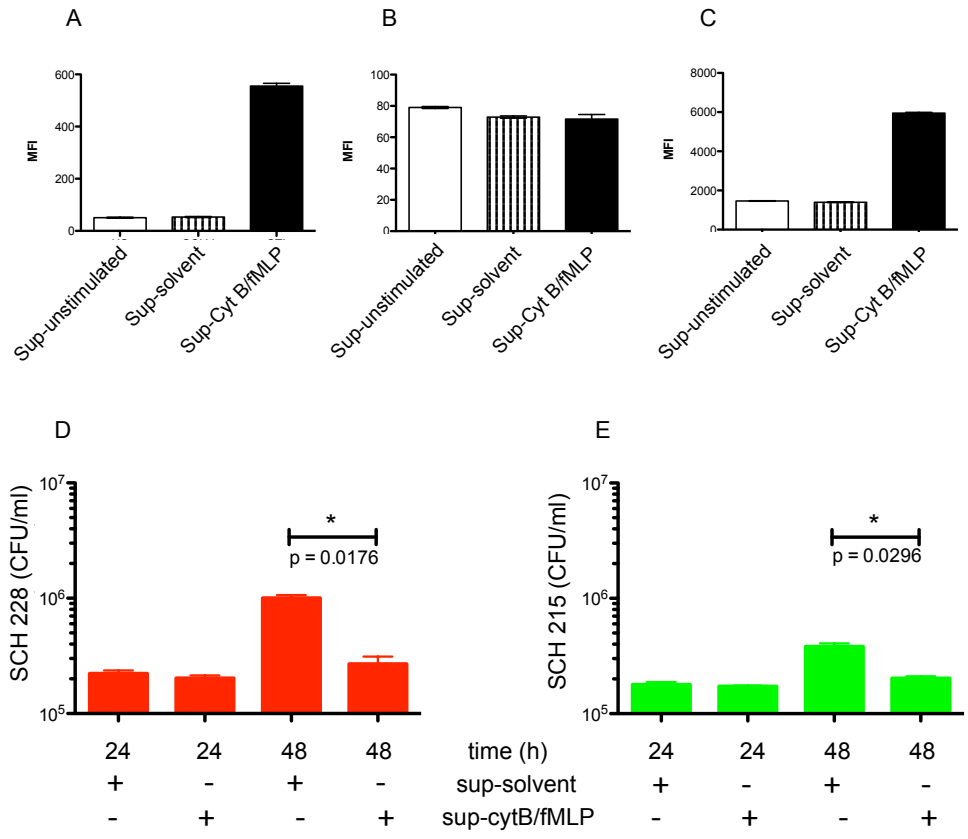


Figure 11 Granular components of human neutrophils inhibit extra-cellular growth.

Neutrophils were incubated with solvents or stimulants, CytB/fMLP for 30 min at 37°C and cells were analyzed for the cell surface expression of granule specific markers CD63 (**A**), CD35 (**B**) and CD66b (**C**) for assessing degranulation of azurophilic, secretory and specific granules, respectively, by flow cytometry. Cells incubated in the TC medium were considered as control. An aliquot (100 µL) of the supernatants from the 10^7 degranulated neutrophils were incubated with 10^5 SCH 228 (**D**) or SCH 215 (**E**) at 24 and 48 h. The effect of supernatants on survival of mycobacteria is shown. Data represent mean±SEM from three different donors. (*p <0.05).

2.12 Supernatant from SCH 228 or SCH 215-infected neutrophils inhibits extracellular growth of *Staphylococcus aureus* but not *M. avium*

Our degranulation study indicates that both SCH 228 and SCH 215 interfere with the degranulation in infected neutrophils. To investigate whether supernatant from SCH 228 or SCH 215 infected neutrophils support extracellular growth of *M. avium*, SCH 228 and SCH 215 were incubated with the supernatant from SCH 228 or SCH 215 infected neutrophils and the survival (CFUs) of these two clinical isolates in the supernatant was determined after 48 h. Supernatant from unstimulated neutrophils was used as control. Although supernatant from cytB/fMLP degranulated neutrophils did affect the viability of both the isolates compared to control supernatant, supernatants from neutrophils infected either with SCH 228 or SCH 215 did not decrease SCH 228 and SCH 215 mycobacterial survival (Fig. 12A and B). These results show that the supernatant from SCH 228 or SCH 215 infected neutrophils does not inhibit the extracellular growth of SCH 228 and SCH 215, and suggest that the supernatant from mycobacteria-infected neutrophils differ in their contents from that of cytB/fMLP induced neutrophils in terms of degranulation.

In order to prove that the contents of the degranulated supernatant from infected neutrophils are different from cytB/fMLP induced supernatant, survival of *S. aureus* (SA), an extracellular pathogen was tested. SA was incubated with the supernatant from cytB/fMLP stimulated, SCH 228, SCH 215 or SA-infected or uninfected neutrophils and its survival in these supernatants was determined after 2 and 4 h by CFU counts (Fig. 12). Supernatant from neutrophils infected with SA did not affect their own survival. Also, supernatant from cytB/fMLP stimulated neutrophils, which had earlier shown to affect mycobacterial extracellular growth, did not affect SA growth in the supernatant (Fig. 12C and D). However, importantly, supernatant from mycobacteria-infected neutrophils significantly prevented SA extracellular growth. Interestingly, supernatant from SCH 215-infected neutrophils prevented SA growth significantly more than SCH 228. These data together demonstrate that supernatants from SCH 228 and SCH 215- infected neutrophils prevent SA extracellular growth and contents as well as antibacterial effects of the degranulated supernatant from neutrophils vary depending upon the stimuli or pathogen.

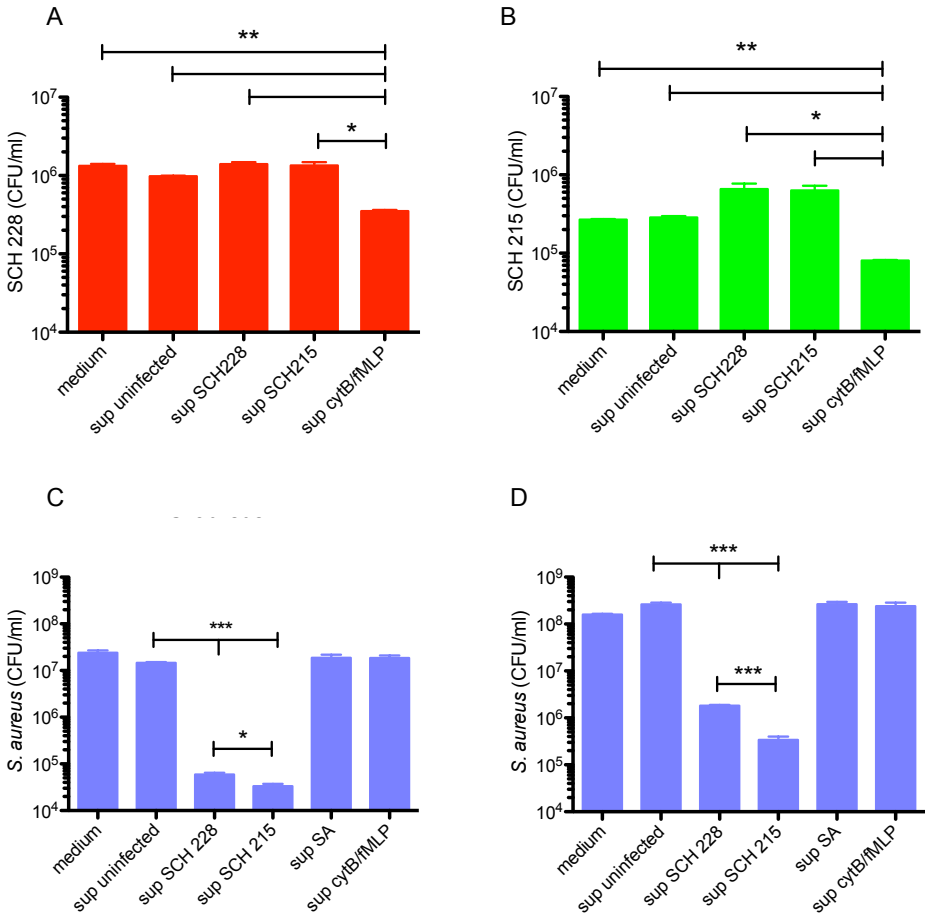


Figure 12 Neutrophil degranulation induced by mycobacteria does not impact on their extra cellular growth but significantly inhibits extra cellular growth of SA.

Neutrophils were incubated with TC medium, SCH 228, SCH 215, SA or CytB/fMLP for 30 min at 37°C. An aliquot (100 µL) of the supernatants from the 10^7 degranulated neutrophils were incubated with 10^5 SCH 228 (**A**) or SCH 215 (**B**) for 24 and 48 h. In other experiments, supernatants were incubated with 10^4 SA for 2 (**C**) and 4 h (**D**). The effect of supernatants on survival of mycobacteria or SA is shown. Data represent mean±SEM from three different donors. (**p* <0.05, ***p* <0.005, ****p* <0.0005).

2.13 LPS-stimulated or unstimulated neutrophils do not affect survival of SCH 228 and SCH 215 in human macrophages in contact co-culture experiments

Neutrophils are persistently recruited to the site of infection and are present in the granulomas in humans (Eum et al., 2010; Martineau et al., 2007). Although neutrophils are terminally differentiated, when stimulated with pathogens or cytokines they can produce cell mediators such as cytokines and chemokines. These cell mediators are known to interact with and have paracrine effects on infected macrophages and aid in the defense against mycobacterial infection. Even though, many studies have suggested that neutrophils contribute to the early defense against mycobacteria, contradictory studies exist (Lowe et al., 2012). Therefore, to evaluate the relevance of the interactions between neutrophils and macrophages in host defense against mycobacterial infection, survival of *M. avium hominissuis* in macrophages was analyzed in co-culture experiments. First, we checked the effect of naïve neutrophils on mycobacterial survival in infected macrophages. Freshly isolated naïve neutrophils were co-cultured with the SCH 228 or SCH 215-infected macrophages and the number of viable bacteria present in the macrophages was determined after 48 and 72 h. At 48 and 72 h, the levels of viable intracellular mycobacteria in macrophages did not differ with or without addition of neutrophils (Fig. 13).

Previous studies have shown that LPS stimulation augments the antimicrobial activity of neutrophils. Therefore, we tested if LPS stimulated neutrophils enhance clearance of intracellular bacteria in the infected macrophages. To this end, we compared the bacterial burden in the infected macrophages cultured in the presence or absence of LPS-stimulated neutrophils. No significant difference in the bacterial burden was observed between the co-culture of unstimulated and LPS-stimulated neutrophils (Fig. 13).

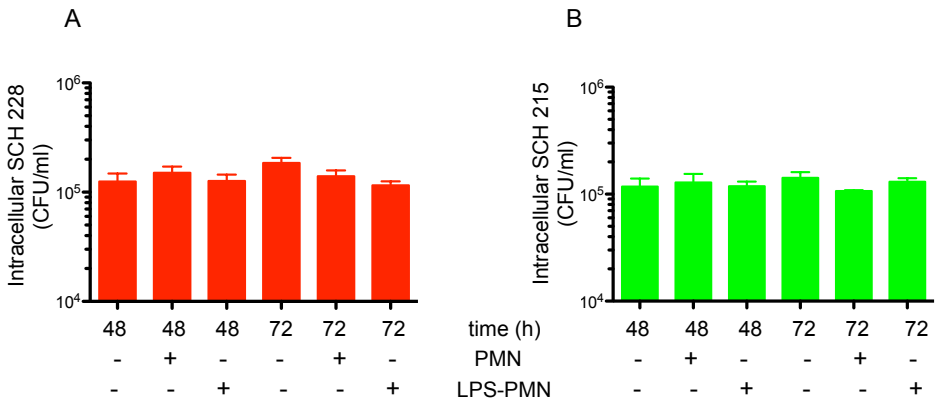


Figure 13 Bacterial survival of SCH 228 and SCH 215 do not differ in human macrophages co-cultured with LPS-stimulated or unstimulated neutrophils.

Mycobacteria-infected human macrophages were incubated with LPS stimulated neutrophils or unstimulated neutrophils at a ratio of 1:1 for 48 and 72 h. At indicated time points bacterial survival of *M. avium* SCH 228 (**A**) and SCH 215 (**B**) was determined by CFUs from cell lysates. Mycobacterial survival in macrophages without neutrophils was considered as control. Data represent mean±SEM from three different donors. (p >0.05, ns).

2.14 SCH 228 and SCH 215-induced degranulation does not affect intracellular survival of these isolates in human macrophages in non contact co-culture experiments

We have shown that SCH 228 and SCH 215 inhibit degranulation in human neutrophils. Moreover, the contents in the supernatant from SCH 228 infected neutrophils differ from SCH 215. Also, we have demonstrated that these contents have the ability to inhibit extracellular growth of *S. aureus*. Hence, we asked whether these contents could affect the intracellular growth of mycobacteria in macrophages. Infected macrophages were grown in the presence of supernatant from uninfected or infected neutrophils and bacterial burden was determined after 48 and 72 h (Fig. 14). Analysis of this non cell-contact co-culture experiment revealed that both supernatants did not affect the intracellular bacterial burden of SCH 228 and SCH 215 in the infected macrophages.

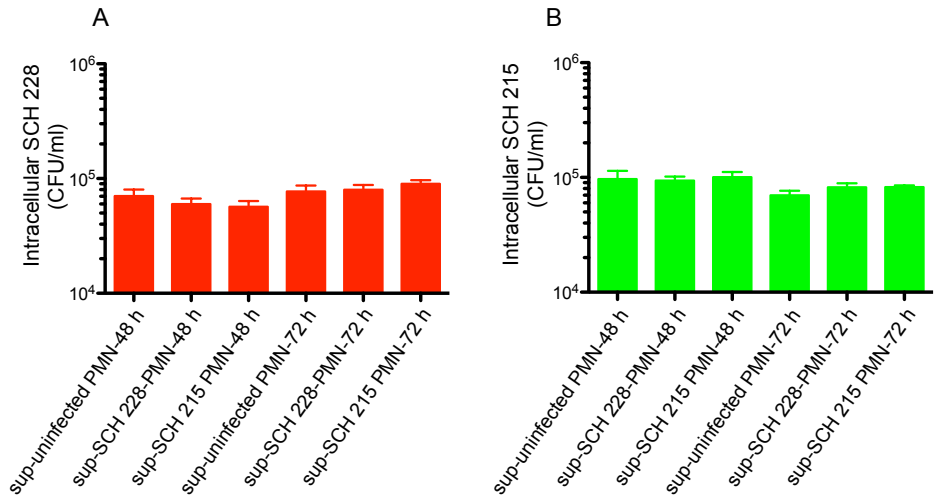


Figure 14 Mycobacteria-induced degranulation does not affect intracellular survival of SCH 228 and SCH 215 in human macrophages.

Mycobacteria-infected human macrophages were incubated with degranulated supernatants for 48 and 72 h. At indicated times, bacterial survival of *M. avium* SCH 228 (**A**) and SCH 215 (**B**) was determined as CFUs from cell lysates. Mycobacterial survival in macrophages incubated with supernatant from unstimulated neutrophils was considered as control. Data represent mean±SEM from three different donors. (p >0.05, ns).

2.15 *M. avium* infected macrophages induce exocytosis of CD66 vesicles predominantly in naïve neutrophils in a time dependent manner

Secretion of anti-microbial peptides and cell mediators from neutrophils is mediated by degranulation, which is inhibited in SCH 228 and SCH 215 infected neutrophils. However, whether infected macrophages could induce degranulation in naïve neutrophils is not known. Thus, we investigated the degranulation of naïve neutrophils upon co-culturing with infected macrophages. Freshly isolated naïve neutrophils were allowed to interact with previously infected macrophages for 30, 60 and 120 min before they were harvested and analyzed for the cell surface expression of the granule specific markers by flow cytometry. The degranulation induced upon the interaction with uninfected naïve macrophages was considered as control. Infected macrophages induced cell surface expression of only CD66b but not CD63 or CD35 in naïve neutrophils upon interaction compared to uninfected macrophages (data not shown). This up-regulation in CD66b expression increased in neutrophils from 30 to 120 min upon interacting with SCH 215-infected macrophages. Such

an increase in CD66b expression in neutrophils that interacted with 228-infected macrophages was observed from 30 to 60 min, but not at 120 min (Fig. 15). These data show that SCH 215 and SCH 228-infected macrophages predominantly induce specific/gelatinase granules release in naïve neutrophils. These data also importantly show that SCH 228-infected macrophages induced CD66b expression in neutrophils only for a shorter time, again highlighting the differential induction of granules by these two clinical isolates.

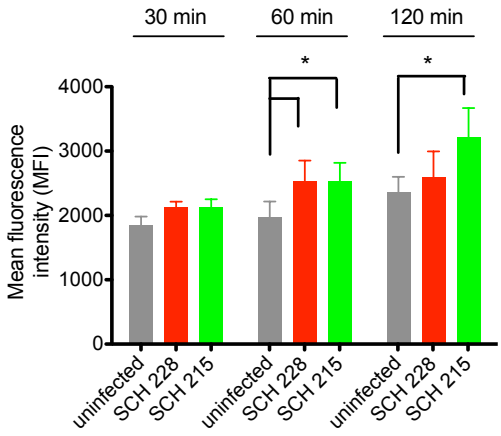


Figure 15 SCH 228 or SCH 215-infected macrophages induce predominantly specific/gelatinase degranulation in naïve neutrophils in a time dependent manner.

Mycobacteria-infected human macrophages were incubated with freshly isolated naïve neutrophils. At indicated time points neutrophils were harvested and analyzed for the cell surface expression of the granule specific marker CD66b for assessing degranulation of specific granules, by flow cytometry. Neutrophils incubated with uninfected macrophages were considered as control (grey line). Red line and green line indicate CD66b on neutrophils co-incubated with SCH 228 and SCH 215 infected macrophages respectively. Data represent mean±SEM from three different donors. (* p <0.05).

2.16 Exosomes released from *M. avium*-infected macrophages attract human neutrophils

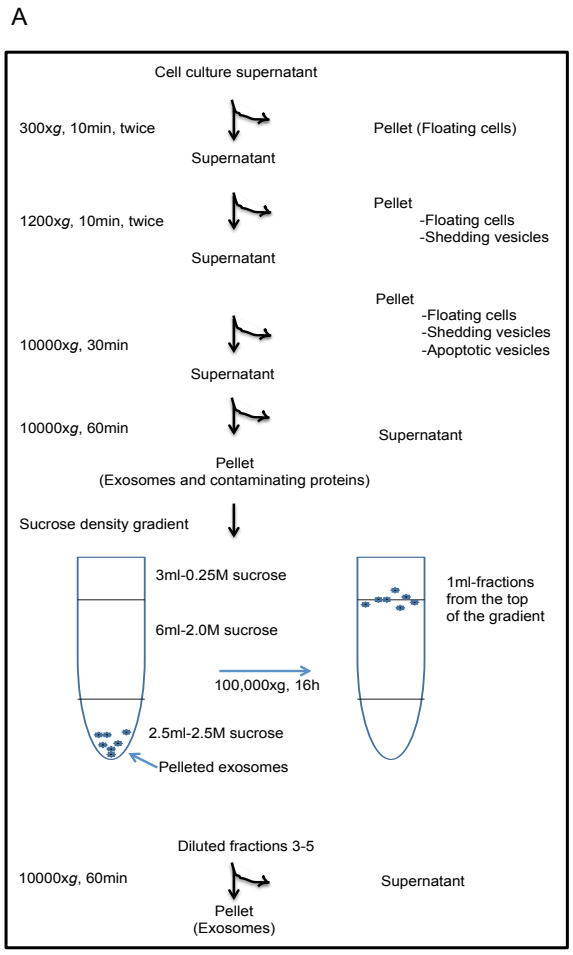
Infected macrophages release cell mediators to attract other immune cells and it is known that neutrophils accumulate at the site of mycobacterial infection (Barrios-Payan et al., 2006; Eum et al., 2010; Feng et al., 2003; Martineau et al., 2007). However, the mechanisms by which neutrophils are attracted to sites of mycobacterial infection are not fully understood. To this end, we evaluated whether *M. avium* strains alone are capable to act as chemo

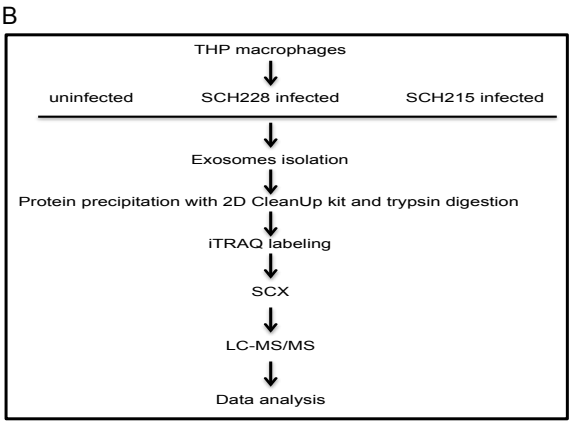
attractants for neutrophils and monocyte/macrophages. We analyzed supernatants from *M. avium* infected monocytes (THP-1 cells) for their ability to attract neutrophils in a Boyden chamber plate migration assay. Reciprocal experiments were performed using supernatants from infected neutrophils to attract THP-1/monocyte derived macrophages (MDM) and vice versa. The results show that supernatants from infected THP-1/MDM exhibit chemo-attractivity towards human neutrophils, but not vice versa (Hartmann, 2006) Interestingly, both intact and heat killed *M. avium* attracted neutrophils. None of the cytokines and chemokines present in the supernatant were alone responsible for the observed chemo-attractivity (Hartmann, 2006). This shows that other factor(s) present in the supernatant probably also in combination of cytokines and chemokines are responsible for the observed chemoattraction. Mass spectrometric (mass spec) analysis of the fraction, which retained the activity, consistently revealed the presence of heat shock protein 70 (HSP70). Since, HSP70 is an exosomal marker, we analyzed whether exosomes have the ability to attract neutrophils. THP macrophages were incubated with TC medium or *M. avium* SCH 228 or SCH 215 for 4 h at 37°C. After incubation, extracellular mycobacteria were removed and cells were incubated for 72 h. Exosomes were then isolated from the supernatants of uninfected or infected THP macrophages using differential centrifugations (Fig. 16A) to remove cells and cell debris. Further purification steps were performed on non-linear sucrose gradient by ultracentrifugation to remove non-membranous proteins, protein aggregates and other larger vesicles. Exosomes were confirmed by presence of exosomal markers Hsp70, Rab5 and 7, and MHC 1 and 2 (Fig. 16C). In addition, the preparations were examined and characterized for the size distribution of exosomes by transmission electron microscopy and were shown to be mostly 20–300 nm in diameter (Fig. 16E).

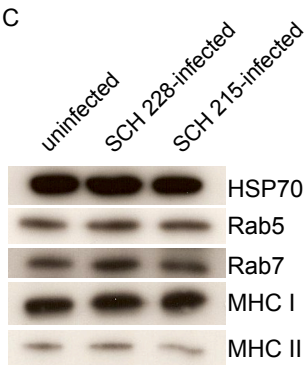
Purified exosomes from uninfected and infected THP macrophages were used as the chemoattractants and checked for their ability to attract Calcein AM-loaded human neutrophils in a Boyden chamber plate migration assay. The migration of neutrophils towards chemoattractants was analyzed by fluorescence measured from the plate and filter of the Boyden chamber. As expected, neutrophils migrated towards Zymosan-activated serum (ZAS), the positive control for this assay. As expected from previous observations, exosomes from infected as well as uninfected macrophages attracted neutrophils. Interestingly, however, the exosomes released from *M. avium*-infected macrophages

attracted substantially higher number of neutrophils than those from uninfected macrophages (Fig. 17). Furthermore, to compare the protein profile of infected vs uninfected THP macrophage derived exosomes, total exosomal proteins were subjected to trypsin digestion, the extracted peptides were purified by strong cationic exchange column chromatography after iTRAQ labeling and the purified peptides were analyzed by nano-LC–MS/MS (Fig. 16B). Protein database searching of MS/MS data resulted in identifying 648 proteins in THP macrophage derived exosomes. Mass spec analysis of the proteins present in the exosomes revealed the presence of also other exosomal markers apart from the aforementioned ones (Fig. 16D). The identified exosomal protein gene symbols were compared with the Exocarta database of exosomal proteins. 22 proteins from our preparation matched with the top 25 proteins that were often identified in exosomal studies as exosomal markers (Fig. 16F). The high overlap of exosomal marker proteins between exosomes from THP macrophages and the Exocarta database suggests that our procedures used for exosomes isolation are reproducible and reliable.

The identified proteins were annotated and classified according to subcellular localization, biological processes and molecular activity. Fig. 18 shows the subcellular localization of the identified proteins based on gene ontology using MASCOT analysis. Our exosome proteome contained proteins that are found to be located in the cytoplasm, intracellular organelles, membranes, cytoplasmic vesicles and other subcellular compartments. In terms of the biological processes as shown in Fig. 18B, among several functions the exosomal proteins are mainly involved in cellular, metabolic and biological processes, response to stimuli, cell communication. The molecular functions of these proteins are shown in Fig. 18C. THP macrophage-derived exosomes are enriched in proteins that have binding, enzyme regulatory and catalytic activities. Apart from host derived proteins, the mass spec analysis of these exosomes also revealed proteins from SCH 228 and SCH 215 *M. avium* (Table 1).

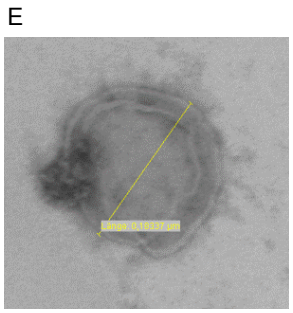






D

Other exosomal markers detected
CD 9
CD 63
CD 81
Syntaxin
LAMP-2
HSC 71
HSP 90
Annexin -1, 2, 4, 5, 6, 7, 11
Tubulin- α and β
Integrin- α and β



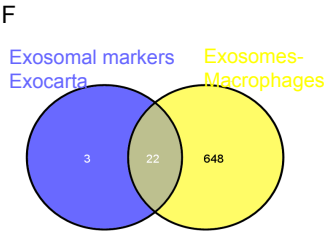


Figure 16 Isolation and characterization of exosomes derived from *M. avium*-infected THP macrophages.

THP macrophages were incubated with TC medium (uninfected) or with *M. avium* (SCH 228 or SCH 215) for 4 h at 37°C. After incubation, extracellular mycobacteria were washed and cells were incubated for 72 h. The culture supernatant was used to isolate exosomes. **A.** Strategy for the isolation and purification of exosomes from culture supernatants based on differential ultracentrifugation. **B.** Workflow for quantitative proteomics and summary of identification and quantification results. **C.** Immunoblot analysis of exosomal markers on exosomes prepared from uninfected or SCH 228 or SCH 215-infected macrophages. **D.** Other exosomal

markers were detected in the mass spec analysis. E. Electron microscopy image of purified exosomes. **F.** Venn diagram showing the overlap in the number of proteins present in the exosomes from macrophages identified by mass spec and the number of exosomal markers listed in the Exocarta database.

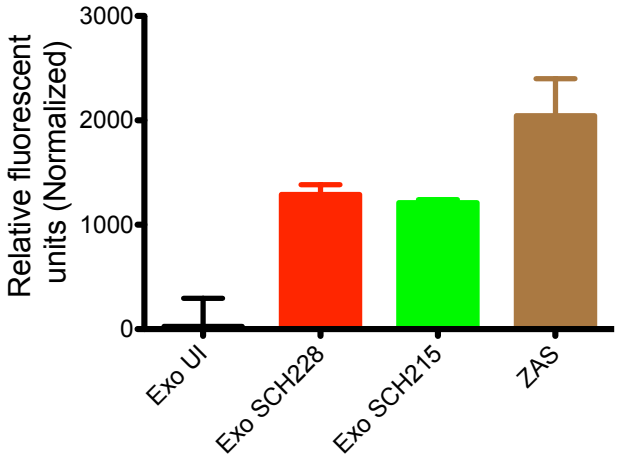
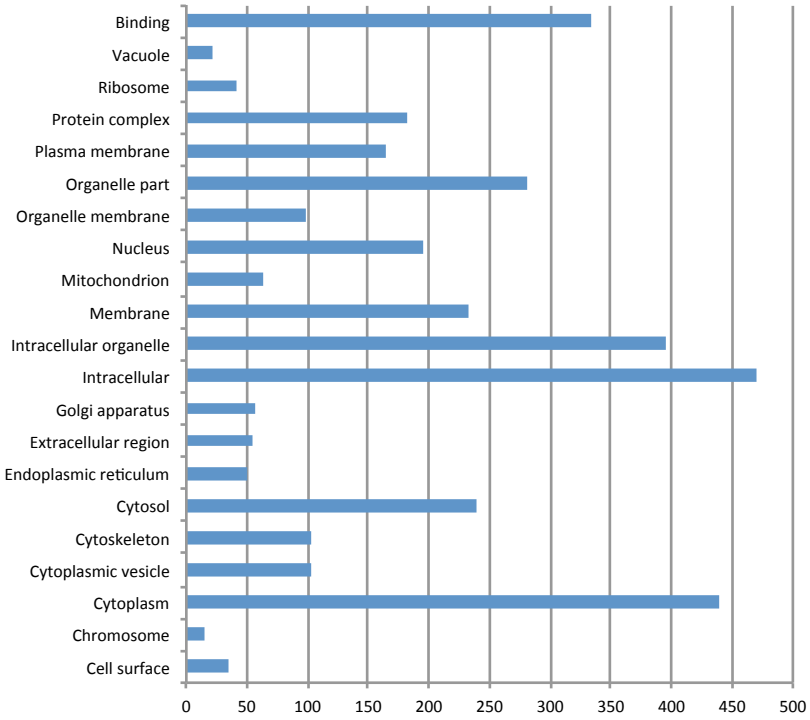


Figure 17 Exosomes released from *M. avium* infected macrophages induce neutrophil chemotaxis.

THP macrophages were incubated with TC medium (uninfected) or with *M. avium* (SCH 228 or SCH 215) for 4 h at 37ºC. After incubation, extracellular mycobacteria were washed and cells were incubated for 72 h. Exosomes were isolated from the respective supernatants using sucrose density gradient centrifugation. Isolated exosomes (50 µg/ml) were checked for their ability to attract human neutrophils using Boyden chamber plate migration assay, as mentioned in the materials and method.

A


Binding
Vacuole
Ribosome
Protein complex
Plasma membrane
Organelle part
Organelle membrane
Nucleus
Mitochondrion
Membrane
Intracellular organelle
Intracellular
Golgi apparatus
Extracellular region
Endoplasmic reticulum
Cytosol
Cytoskeleton
Cytoplasmic vesicle
Cytoplasm
Chromosome
Cell surface
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50
100
150
200
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400
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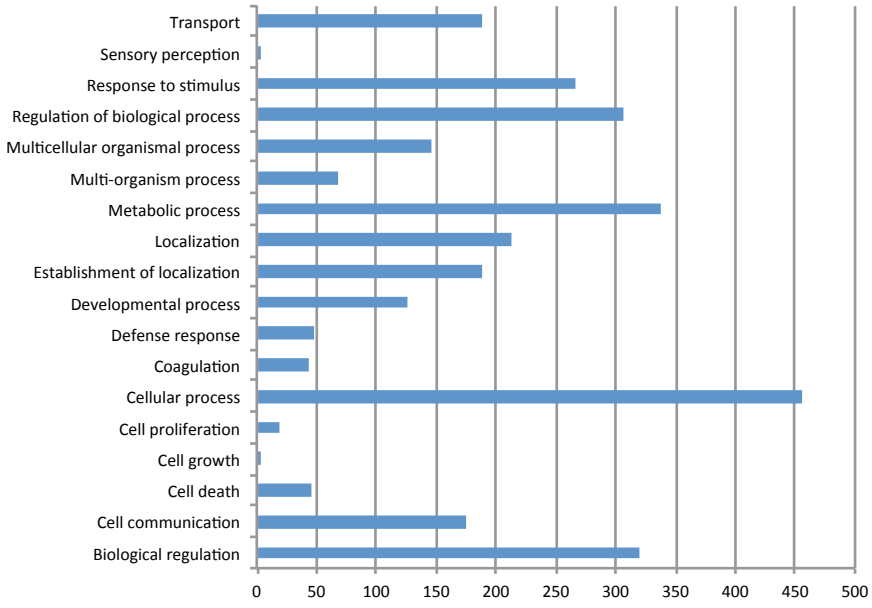
500

B


Transport
Sensory perception
Response to stimulus
Regulation of biological process
Multicellular organismal process
Multi-organism process
Metabolic process
Localization
Establishment of localization
Developmental process
Defense response
Coagulation
Cellular process
Cell proliferation
Cell growth
Cell death
Cell communication
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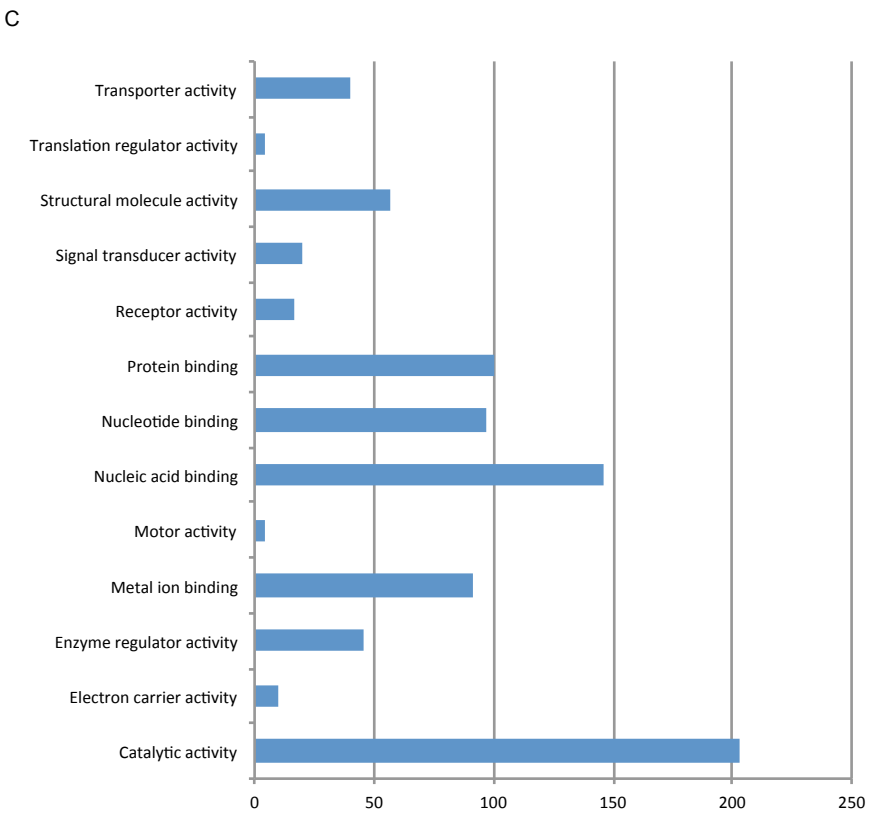

Figure 18 Classification of identified proteins present in the exosomes derived from THP-1 macrophages.

Proteins present in the exosomes were identified by mass spec analysis. Classification of identified proteins by subcellular localization (A), processes (B) and activities (C) was done based on GO analysis using MASCOT.

2.17 Prediction of putative neutrophil chemotactic factors on THP-macrophage derived exosomes

Having shown the ability of exosomes in attracting neutrophils, we employed mass spec data to predict putative candidates for neutrophil chemotaxis. For this purpose, we performed detailed GO enrichment analysis of the proteins involved in cell communication. Out of the proteins involved in cell communication, we next sorted out proteins playing a role in cell migration and neutrophil chemotaxis. Table 2 shows the putative proteins on exosomes that participate in neutrophil chemotaxis. Proteins having a peptide score more than 40 and the amount of which was more than that present in exosomes released from uninfected THP macrophages were searched in GO, Exocarta, PUBMED and UniProt databases.

Interestingly, all of these candidate proteins were previously reported to be present on exosomes (Table. 2). Analysis based on PUBMED search revealed that these proteins have been shown to be involved either directly or indirectly in migration of cells including neutrophils. Together, the presence of several potential putative chemoattractants on exosomes derived from *M. avium*-infected THP macrophages supports the result of our functional analysis that these exosomes are capable of attracting human neutrophils.

Table 1 List of mycobacterial proteins present in exosomes

Accession	Protein	Mw (kDa)
gi|516826014	30S ribosomal protein S6 [Corynebacterium mastitidis]	11.3
gi|492831729	6-phosphofructokinase [Actinomyces graevenitzii]	80.5
gi|386739738	adenylate kinase [Corynebacterium pseudotuberculosis 31]	16.5
gi|496916762	ATPase/histidine kinase/DNA gyrase B/HSP90 domain protein [Actinomyces sp. oral taxon 181]	30.8
gi|118467500	dimethylmenaquinone methyltransferase [Mycobacterium smegmatis str. MC2 155]	22.6
gi|491840837	glyceraldehyde-3-phosphate dehydrogenase [Mycobacterium hassiacum]	35.3
gi|495047224	haloalkane dehalogenase [Mycobacterium colombiense]	33.1
gi|491285106	hypothetical protein [Mycobacterium rhodesiae]	66.6
gi|489996938	hypothetical protein, partial [Mycobacterium tuberculosis]	17.2
gi|433646793	LOW QUALITY PROTEIN: adenylate kinase-like kinase [Mycobacterium smegmatis JS623]	19.9
gi|517431797	membrane protein [Mycobacterium sp. 155]	39.4
gi|41410362	molecular chaperone	10.7

	GroES [Mycobacterium avium subsp. paratuberculosis K-10]	
gi|497429212	phosphatase [Actinomyces sp. oral taxon 178]	33.8

Table 2 List of putative proteins on exosomes involved in neutrophil chemotaxis

UniProt ID [a]	**Protein name** [a]	**MW** [b] **(kDa)**	**Location** [c]	**Reported on Exosomes** [c]	**Involved in cell migration**
ANXA4	Annexin A4 (Endonexin-1)	35.9	-Extracellular vesicular exosomes -Cytoplasm	(Buschow et al., 2010; Gonzales et al., 2009; Gonzalez-Begne et al., 2009)	(Mogami et al., 2013)
ITA5	Integrin alpha 5 (CD49e-Fibronectin receptor subunit alpha) (VLA5-very late antigen 5)	114.5	-Membrane -Single-pass type1 membrane -Cytoskeleton	(Ji et al., 2008)	(Burns et al., 2001; van den Berg et al., 2001)
ITAX	Integrin alpha X (CD11c)	127.7	-Membrane -Single-pass type1 membrane -Cytoskeleton		
UPAR	Urokinase plasminogen activator surface receptor (CD87-monocyte activation antigen Mo3)	37.0	-Membrane -Secreted	(Jung et al., 2009; Mu et al., 2013)	(Dekkers et al., 2000; Gyetko et al., 1995; Liu et al., 2011; Mukhina et al., 2000; Pliyev et al., 2011)

ANXA1	Annexin A1	38.7	-Membrane -Cytoplasm -Extracellular vesicular exosomes	(Kesimer et al., 2009; Stamer et al., 2011)	(Dalli et al., 2012; Walther et al., 2000)
SDCB1	Syntenin-1	32.4	-Membrane -Cytoplasm -Cytoskeleton -Extracellular vesicular exosomes	(Mears et al., 2004; Stamer et al., 2011)	(Kim et al., 2014; Sala-Valdes et al., 2012)
ITAV	Integrin alpha V (CD51-Vitronectin receptor subunit alpha)	116.0	-Membrane -Single-pass type1 membrane -Extracellular vesicular exosomes	(Buschow et al., 2010; Gonzales et al., 2009)	
CD97	CD97 antigen (Leukocyte antigen)	91.8	-Membrane -Secreted -Extracellular vesicular exosomes	(Buschow et al., 2010; Turiak et al., 2011)	(Hamann et al., 2010; Leemans et al., 2004; Veninga et al., 2008; Wang et al., 2007)
CD44	CD44 antigen	81.5	-Membrane -Cytoplasm -Extracellular vesicular exosomes	(Buschow et al., 2010; Gonzalez-Begne et al., 2009)	(Brazil et al., 2010; Khan et al., 2004; Zech et al., 2012)
BASI	Basigin (CD147-leukocyte activation antigen M6) Extracellular matrix metalloproteinase inducer	42.2	-Extracellular vesicular exosomes -Membrane -Single-pass type1 membrane	(Buschow et al., 2010)	(Damsker et al., 2009; Kato et al., 2009; Lu et al., 2010)
CD9	CD9 antigen (Leukocyte antigen MIC3)	25.4	-Extracellular vesicular exosomes -Membrane	(Gonzales et al., 2009; Gonzalez-Begne et al.,	(Powner et al., 2011)

			-Single-pass type1 membrane	2009; Pisitkun et al., 2004)	
ADA10	Disintegrin and metalloproteinase domain-containing protein 10 (CD156c)	84.1	-Extracellular vesicular exosomes -Membrane -Single-pass type1 membrane -Cytoplasm	(Buschow et al., 2010; Padro et al., 2013; Stoeck et al., 2006)	(Blume et al., 2012; Schulz et al., 2008; Schwarz et al., 2010)
CD45	Leukocyte common antigen	147.2	-Extracellular vesicular exosomes -Membrane -Single-pass type1 membrane	(Buschow et al., 2010; Wubbolts et al., 2003)	(Harvath et al., 1991; Zhu et al., 2011)
LEG1	Galectin-1 (Putative MAPK-activating protein PM12)	14.7	-Cytoplasm -Secreted	(Welton et al., 2010)	(Auvynet et al., 2013)
LEG3	Galectin-3 (Mac-2 antigen)	26.1	-Extracellular vesicular exosomes -Cytoplasm -Secreted	(Gonzales et al., 2009; Gonzalez-Begne et al., 2009; Thery et al., 2001; Welton et al., 2010)	(Bhaumik et al., 2013; Sano et al., 2000)
LEG9	Galectin-9	39.5	-Cytoplasm -Secreted	(Keryer-Bibens et al., 2006; Koumangoye et al., 2011)	

a – based on Gene Ontology (GO) search; more than 40 peptide score; manually cured for their amount
b – based on MASCOT analysis
c – based on UniProt, GO, Exocarta and PUBMED search

3 Discussion

Most of the investigations regarding the understanding of the pathophysiological behavior of *M. avium* are carried out using type strains or laboratory strains. However, naturally occurring *M. avium* infections are caused by strains that are widely different in genotype and phenotype. Very little is known about the characteristics of these clinical isolates in terms of basic biology, virulence and *in vivo* pathogenicity. The genetic variability of mycobacterial strains might lead to sharp phenotypic differences, which could be responsible for a differential modulation of host immune responses impacting in the onset and progression of the disease (Janulionis et al., 2005; Li et al., 2002). The underlying host-pathogen interactions that account for distinct clinical characteristics observed in patients suffering from *M. avium* infections are largely unknown. Previously, our group has showed that human neutrophils are able to phagocytize and kill the avian type strain, *Mycobacterium avium* TMC 724, *in vitro* (Hartmann et al., 2001). However, whether human neutrophils can kill *M. avium* isolated from human patients is not known. In this study we used clinical isolates of *M. avium hominissuis* (SCH 228 and SCH 215). SCH 228 was isolated from the bone marrow of an AIDS patient suffering from disseminated *M. avium* infection while SCH 215 originated from a toddler suffering from lymphadenitis coli. These strains are of differential virulence as shown by their highly differential growth rate in macrophages (Meyer et al., 1998). Here we have investigated how the differential virulence of these two human clinical isolates impacts on cell-autonomous immune regulation of neutrophils.

3.1 Killing of *M. avium*

The fact that human neutrophils could phagocytize and kill the avian type strain, *Mycobacterium avium* TMC 724, (Hartmann et al., 2001) led us to ask whether human neutrophils could kill human clinical *M. avium hominissuis* isolates, SCH 228 and SCH 215. Although human neutrophils kill TMC 724, they could not kill the SCH 228 and SCH 215 (Fig.1). In particular there was no difference in bacterial uptake and survival within neutrophils between these two strains suggesting that virulence affects neutrophil autonomous defense. Several *in vitro* studies and animal infection models reveal that phylogenetic diversities among Mtb strains as well as non-tuberculous strains exhibit

different virulence and pathogenesis phenotypes in macrophages (Amaral et al., 2011; Lopez et al., 2003; Manabe et al., 2003; Manca et al., 2004; Manca et al., 2001; Palanisamy et al., 2009; Vrba-Pech et al., 2013). It is thus likely that the clinical isolates SCH 228 and SCH 215 that had been challenged in human patients may show different virulence and pathogenic phenotypes compared to TMC 724. Corleis et al. (2012) reported that human neutrophils phagocytize and kill *M. smegmatis*, a non-tuberculous mycobacteria within 3 h, supporting our previous published results where we showed that neutrophils were capable of killing non-tuberculous mycobacteria. Furthermore, they showed that though human neutrophils could phagocytize, virulence of Mtb type strain H37Rv impact the killing ability of the neutrophils.

A protective role for neutrophils in mycobacterial infection in vivo has been addressed in mouse studies. Barrios-Payan et al. (2006) reported that granulocyte receptor 1 (GR-1) antibody-mediated depletion of granulocytes in mice before intratracheal infection with Mtb increases the number of CFU subsequently recovered from lungs and spleen. On the other hand, using similar antibody-mediated depletion of granulocytes, Seiler et al. (2000) showed that neutrophils have no effect on the bacterial burden of mice infected intravenously with 10^6 CFU of Mtb, *M. bovis* BCG or the non-tuberculous strain *M. fortuitum*. However anti-GR-1 is not specific for neutrophils. Thus the depletion of (Gr-1) positive cells may have off target effects such as targeting Ly6C on plasmacytoid dendritic cells (DCs), monocytes and CD8 positive T cells (Wojtasiak et al., 2010). Overall studies addressing the killing capacity for neutrophils *in vivo* and *in vitro* provide controversial data that may be attributed to the high variability in the methods used.

3.2 Phagosomal processing

Virulent mycobacteria are able to survive and propagate inside macrophages by interfering with the phagosomal maturation process (Rohde et al., 2007a; Rohde et al., 2007b; Russell, 2007; Russell et al., 2010a; Sweet et al., 2010). In macrophages, the sequential maturation of phagosomes including acidification is required for the fusion of lysosomes with phagosomes in order to kill pathogens (Cougoule et al., 2002; Deretic et al., 2006; Desjardins et al., 1994;

Nusse, 2011; Via et al., 1997; Vieira et al., 2002). Similarly, in neutrophils, maturation of phagosomes requires the fusion of granular contents with phagosomes prior to the fusion of lysosomes with phagosomes for digestion of pathogens (Nordenfelt and Tapper, 2011; Segal, 2005; Smith, 1994; Urban et al., 2006; Winterbourn et al., 2006; Winterbourn and Kettle, 2013). Phagosomal maturation can be studied using C12-FDG, as its fluorescence increases by the cleavage of galactopyranoside by β-galactosidase, a marker of lysosomal activity. This activity reflects the fusion of lysosomes with phagosomes and can be studied using flow cytometry (Robinson et al., 2008). In the present report, we have investigated whether phagosomal maturation in infected neutrophils was inhibited by these two clinical isolates. Fluorescence of C12-FDG coated mycobacteria did not increase at all till 5 h (Fig. 2A and B) while the coated magnetic beads, the positive control, showed a clear shift in the fluorescence (Fig. 2C), indicating that fusion of lysosome with phagosomes is inhibited in neutrophils infected with these isolates. Our report is in agreement with many other studies (Cougoule et al., 2002; N'Diaye et al., 1998). N'Diaye et al. (1998) reported in their study that mycobacteria can uncouple phagocytosis from fusion of granules, especially azurophilic granules, which may potentially affect the maturation of phagosomes in infected neutrophils. Such inhibition of phagolysosomal fusion in infected macrophages is a key feature of mycobacterial survival (Deretic et al., 2006; Russell, 2001, 2007; Russell et al., 2010b). Mtb secretes protein tyrosine phosphatase (PtpA), to inhibit acidification of phagosomes, as a mechanism to prevent phago-lysosomal fusion (Wong et al., 2011). In addition to proteins associated with virulence, lipid components of the mycobacterial surface also contribute to the arrest of phagolysosome fusion in macrophages (Robinson et al., 2008; Russell, 2001; Russell et al., 2010b). Increased fluorescence of C12-FDG from heat-killed mycobacteria compared to that of C12-FDG coated live mycobacteria suggests that phagosomes containing heat-killed mycobacteria mature into phagolysosomes in infected neutrophils (Fig. 2D). Proteins associated with virulence of these isolates would have denatured upon heat inactivation but not lipid components. These lipid components may still play a role in inhibition of phago-lysosomal fusion. Even though the heat inactivated isolates showed increased fluorescence than viable bacteria, their shift in fluorescence is much lesser than that of coated beads. These data also reflect the fact that lipid components of these isolates are able to inhibit phagosomal maturation.

3.3 Neutrophil activation

Neutrophils mostly rely on toll-like receptors (TLRs) for their pathogen recognition capacities, and stimulation of these receptors usually leads to the activation MAPKs, PI3K, NF-κB, and AP-1 (Akira and Takeda, 2004; Aleman et al., 2004; Hayashi et al., 2003; Janssens and Beyaert, 2003; Parker et al., 2005; Prince et al., 2011; Sabroe et al., 2005). Regulation of neutrophil functions requires the activation of protein kinase C (PKC) and activation of protein tyrosine kinases (Perskvist et al., 2000; Smith, 1994). This activation leads to the phosphorylation-mediated activation of different phospholipases and kinases, e.g., Shc protein, phospholipase, PI3K, and MAPK (Perskvist et al., 2000; Sue et al., 1997; Zu et al., 1998). Importantly, these intracellular signals that are involved in neutrophil activation are potent modulators of neutrophil microbicidal responsiveness, such as ROS and granule secretion (Brumell et al., 1996; Sue et al., 1997). We found that both SCH 228 and SCH 215 dampened the activation of p38 MAPK in the early times of infection, but was later maintained at a moderate level at 2 h (Fig. 3). These data suggest that such an initial down regulation of p38 MAPK is the key evasion mechanism that may promote these isolates to survive in neutrophils by evading killing mechanisms such as ROS and degranulation. In fact, many studies have concluded that p38 MAPK plays an important role in the innate and adaptive immune response, and several studies have implicated p38 MAPK as a signaling target for mycobacterial infections (Peng et al., 2011; Reiling et al., 2001; Surewicz et al., 2004). Of note, these clinical *M. avium* isolates dampened p38 MAPK activation in neutrophils at least in the early time points, whereas, on the contrary others have shown that *M. avium* activates this pathway to interfere with killing by macrophages (Pathak et al., 2004; Tse et al., 2002; Yadav et al., 2004). Activation of p38 MAPK in infected macrophages leads to SOCS proteins (negative regulators of cytokine signaling)-mediated manipulation of IFN-γ responses (Vazquez et al., 2006). Also, p38 MAPK mediated IL-10 expression influences the acidification of *M. avium* containing phagosomes (Fratti et al., 2003; Souza et al., 2007; Weiss et al., 2002). Binding of *M. avium* to TLR-2 is crucial for the p38 MAPK mediated suppression of antimicrobial responses within phagocytes (Weiss et al., 2008). Recently, it has been found that surface components of mycobacteria are involved in the manipulation of macrophage host defense mechanisms by differentially activating TLRs (Souza et al., 2013). Interestingly, initial transient activation

of p38 MAPK is a key mechanism that enables *M. avium* to survive in bovine monocytes (Fratti et al., 2003; Souza et al., 2007), whereas, in our study, it is the opposite, that is, initial down regulation of p38 MAPK activation in human neutrophils enables the survival of these two clinical *M. avium* isolates. It has been reported that such a limited activation of neutrophils by *M. abscessus*, a member of NTM enables the pathogen to promote its survival (Malcolm et al., 2013). This dichotomy in immune evasion of *M. avium* in neutrophils and macrophages can potentially be attributed to the modifications in the cell envelop components of mycobacteria such as glyco-peptidolipids, phenolic glycolipids, etc. (Brightbill et al., 1999; Robinson et al., 2008; Russell et al., 2010b; Sweet and Schorey, 2006; Sweet et al., 2010; Sweet et al., 2008).

3.4 Degranulation

Secretion of granular contents into the pathogen containing phagosomes is instrumental for intra-phagosomal killing in neutrophils. However, neutrophil degranulation upon mycobacterial infection was not studied in detail till date. There are four types of granules present in the neutrophils; azurophilic, specific, gelatinase and secretory granules. Each of these granules has some components unique to themselves, whereas some others are present in other granules as well. Upon binding of pathogens to receptors, neutrophils initiate a first wave of granules release mainly from secretory granules (Bentwood and Henson, 1980; Faurschou and Borregaard, 2003; Lacy, 2006). These granules provide preformed receptors required on the surface of neutrophils for their activation. On further stimulation from the pathogen-TLR interactions, another wave of granule release happens. Engagement of pathogens with TLRs induces release of granules, which is mediated through p38 MAPK pathway (Appelberg, 2007; Binet and Girard, 2008; Mocsai et al., 2000; Sengelov et al., 1993; Zhong et al., 2003). An inhibition of fusion of azurophilic granules with mycobacteria-containing phagosomes in neutrophils has been described and attributed to the inhibition of TLR2-Hck-p38 MAPK activation pathway (Mocsai et al., 2000; Mocsai et al., 1999; N'Diaye et al., 1998). In our study we not only find a dampened p38 MAPK activation (Fig. 3) but also release of secretory granules does not exceed the amount that is induced upon fMLP stimulation of neutrophils that have been pretreated with a p38MAPK-inhibitor

(Fig. 4B). We conclude from these data that both clinical isolates, SCH 215 and SCH 228, dampen degranulation by inhibiting p38MAPK, which subsequently results in a compromised activation of neutrophils.

Phagosomal killing of pathogens begins when contents from granules are fused with phagosomes, especially contents from both azurophilic and specific granules. Azurophilic granules contain lysosomal enzymes such myeloperoxidase (MPO), bactericidal permeability-increasing protein, defensins, cathepsin G (a family of serine proteases), neutrophil elastase and proteinase 3. Specific granules contain a battery of antimicrobial substances (e.g. alkaline phosphatases, collagenases, lactoferrin, lipocalin (NGAL), lysozyme, and hCAP18, the proform of LL-37). Contents from azurophilic as well as specific granules are released either into the phagosome or to the exterior of the cells (Borregaard, 2010; Borregaard et al., 2007; Faurschou and Borregaard, 2003). Here, we reported that both of these isolates inhibited the release of specific and secretory granules (Fig. 4). The release of azurophilic granules from mycobacteria infected neutrophils could neither be distinguished from uninfected nor from fMLP stimulated neutrophils. This may be due to the fact that these granules are low in abundance and are generally fused with the phagosomes. Our data suggest that one of the mechanisms by which both of these isolates evade killing by neutrophils is the inhibition of neutrophil degranulation.

3.5 ROS

We found that SCH 228 and SCH 215 clinical isolates induced differential ROS production in human neutrophils; SCH 215 induced significantly higher ROS levels than SCH 228 (Fig. 5). Neutrophil NADPH oxidase (NOX-2) plays a key role in host defense and in inflammation by releasing large amounts of ROS (Dupre-Crochet et al., 2013). Upon activation by pathogens, neutrophils induce the assembly of NOX-2 complex on the phagosomal membrane, and, Rac-2 activation is important for this assembly. Activation of Rac-2 requires signals from TLRs and other pathogen recognizing receptors that involve protein tyrosine kinase activity. Also, production of ROS involves the activation of p38

MAPK (Makni-Maalej et al., 2013). Since, we observed that these two isolates differentially dampened the activation of p38 MAPK (Fig. 3), it was likely that they would induce differential ROS production in human neutrophils. Indeed, as expected, SCH 228 induced significantly lower levels of ROS as compared to SCH 215, due to its relatively higher efficiency in dampening the p38 MAPK activation (Fig. 5A). This was further confirmed by measuring ROS from neutrophils co-infected with both these isolates, where the level of ROS induction by SCH 215 was reduced in presence of SCH 228 (Fig. 5C). The significant reduction in ROS is not due to cell death, as the viability of cells was found to be more than 85%, as determined microscopically by trypan blue staining of neutrophils. On one hand, one may postulate that owing to their differential envelop modifications, SCH 228 is able to reduce p38 MAPK-mediated ROS as compared to SCH 215. On the other hand, one may predict that SCH 228 hampers ROS induction because it is more susceptible and may be killed by ROS. However, in our hands, this was not the case: SCH 228 was not killed by neutrophils that are either activated by ROS-inducing fMLP or activated by exogenous ROS (data not shown). Even though, SCH 215 induced significantly higher levels of ROS in neutrophils, none of these isolates are killed (Fig. 1). These data suggest that these isolates have developed differential mechanisms to evade ROS-mediated killing. It has been demonstrated that glyco-peptidolipids from *M. avium* can scavenge ROS in macrophages (Scherer et al., 1997). Recently, McNamara et al. (2013) reported the proteomic characterization of surface exposed proteins from *M. avium* 104 , a clinical strain and reference strain of *M. avium hominissuis* and the identification of a surface exposed virulence associated protein, Cu-Zn SOD. Using a knockout strain, they suggested that a role for Cu-Zn SOD in the survival of *M. avium* in human neutrophils. Also, secretion of virulence factors from Mtb has been reported to have ROS scavenging functions in macrophages to prevent killing (Velmurugan et al., 2007). Similar to our observation, Romero et al. (2012) reported that human clinical Mtb isolates showed differential abilities in inducing ROS in neutrophils. Whether ROS alone is sufficient to kill pathogens, is still under investigation (Dupre-Crochet et al., 2013; Mocsai, 2013; Parker et al., 2012). The fact that the significant difference in ROS induction comparing SCH 215 with SCH 228 has no impact on the bacterial survival of these two strains suggests that oxidative mechanisms have no role in the killing of these two particular clinical isolates.

The process of phagocytosis of pathogens by neutrophils is accompanied by release of ROS at high levels, a feature known as respiratory burst. After formation of phagosomes, ROS has to return to basal levels in order for the phagosomes to acidify (Segal, 2005). If ROS accumulates in the phagosomes, it would consume considerable amount of protons, which are pumped into and required for acidification of the phagosomes. This would consequently increase the pH of the phagosomes (Winterbourn and Kettle, 2013). In order to employ acidification of the phagosomes, ROS is assimilated or scavenged by anti-oxidant systems such as super oxide dismutase (SOD). Upon scavenging of ROS, the pH drops gradually due to movement of various ions into the phagosomes. This acidification is essential for the fusion of various granules and lysosomes with phagosomes for efficient killing of pathogens (Segal, 2005; Winterbourn et al., 2006; Winterbourn and Kettle, 2013). In fact for macrophages it has been shown that increase in the intracellular levels of glutathione (GSH), another ROS scavenger, reduced levels of free radicals in macrophages and thereby, increased the acidification of Mtb containing phagosomes resulting in effective killing of Mtb Morris et al. (2013).

Our ROS kinetics revealed that for these two isolates even when ROS declines after having reached its peak concentration, it never falls to basal levels but is induced in moderate amounts in neutrophils up to 4 h of infection (Fig. 5A). It is possible that this continuous low-level induction of ROS throughout the infection prevents acidification of mycobacteria-containing phagosomes, however, this hypothesis needs further proof.

3.6 Cytokines

Induction of cytokines upon pathogen binding to TLRs is mediated by p38 MAPK (Makni-Maalej et al., 2013; Zu et al., 1996; Zu et al., 1998). As predicted, neutrophils infected with *M. avium hominissuis* SCH 215 and SCH 228 clinical isolates, did not induce most of the cytokines known to be released upon neutrophil activation. However, we observed differential induction of IL-8 and IL-1ra, SCH 228 being the least inducer of these cytokines (Fig. 6), and this correlates with the differential inhibition in p38 MAPK activation by these two isolates (Fig. 3). Importantly, these two isolates literally abolished induction of pro-

inflammatory cytokines such as TNF- α, IFN- γ, IL-6 and IL-1 β, presumably by dampening TLR2-p38 MAPK pathway. It has been reported that activation through TLR and p38 MAPK is important for many functions including production of pro-inflammatory cytokines in immune cells. (Trinchieri and Sher, 2007; Zu et al., 1996; Zu et al., 1998). One may propose that induction of anti-inflammatory cytokines, such as, IL-10, inhibited the pro-inflammatory cytokine production in neutrophils infected with these isolates. However, this is not the case, as these isolates did not induce IL-10 production as well. Type strain Mtb has been shown to induce lower levels of TNF-α, IL-6 andIL-8 in human neutrophils than NTM (Faldt et al., 2002). Portevin et al. (2011) reported that clinical isolates of Mtb showed variation in the induction of pro-inflammatory cytokines and chemokines in macrophages. Others have shown that *M. avium. hominissuis* induced more of anti-inflammatory and less of pro-inflammatory cytokines in human macrophages when compared to *M. avium avium* (Thegerstrom et al., 2012).

The fact that in the time line of neutrophil infection with our two strains ROS is released earlier than IL-8, suggests that in addition to p38 MAPK, ROS may also be involved in the induction of IL-8. Indeed, we could show by using a ROS scavenger, SOD, that ROS is involved in the induction of IL-8 (Fig. 7B). The involvement of ROS in IL-8 induction indicates that it may be acting as a signaling molecule. Our experiments with exogenous addition of ROS confirmed the role of ROS as a signaling molecule (Fig. 7C). Furthermore inhibition of p38MAPK, significantly reduced ROS (Fig.5B) as well as IL8 release (Fig. 7A) from neutrophils infected with either of our two clinical isolates. Together, our data indicate that p38 MAPK and ROS are involved in the induction of IL-8 in neutrophils infected with SCH 215 and SCH 228. In fact, it is known that as a signaling molecule, oxidative stress by ROS can activate MAPKs, including p38 MAPK. However, the mechanisms by which ROS can activate these kinases are unclear. It is also not clear where ROS is located and where ROS binds to exert their signaling functions including p38 MAPK activation (D'Autreaux and Toledano, 2007; Finkel, 2011; Son et al., 2011; Tonks, 2005). As mentioned above, both isolates maintained ROS induction on low levels even after declining from its peak concentration and still ROS had no impact on survival of these isolates. Presumably a sustained induction of ROS would maintain the activation of various MAPKs, including p38.

Our western blot results show that, these isolates dampened the receptor-mediated p38 MAPK activation, which can be appreciated from 15 min p.i (Fig. 3). The fact that the ROS induction is delayed till 30 min and p38 MAPK activation was restored gradually after 2 h, suggest that ROS may be involved in the activation of p38 MAPK later in the time course of infection. Recently, many reports have described the involvement of ROS-mediated p38 MAPK activation in cytokine induction, including IL-8 (Boggaram et al., 2013; Lee et al., 2009; Liu et al., 2014; Mitra and Abraham, 2006). Treatment of neutrophils with IL-8, which binds to CXCR1/2 receptors, induces release of the granules including azurophilic granules (Fig. 8), which reflects the fact that activation of receptor-mediated p38 MAPK is required for the efficient release of granular contents. However, activation of neutrophils with either IL-8 (data not shown) or the TLR 4 agonist LPS, did not affect the killing capacity of human neutrophils (Fig. 9), highlighting the fact that these clinical *M. avium* strains down regulate the activation of receptor-mediated p38 MAPK which is required for killing in human neutrophils. These data collectively indicate that activation of receptor-mediated p38 MAPK is actually detrimental to the survival of these isolates in neutrophils but not the ROS-mediated p38 activation. Extrapolating this data may suggest that ROS as a signaling molecule may not bind to TLR receptor to activate p38 MAPK.

3.7 Cell death

Prevention of pathogen induced cell death is part of the immune evasion mechanisms of these isolates in neutrophils. We observed that these isolates neither induced apoptosis nor necrosis (Fig. 10). It has been postulated that apoptotic neutrophils containing mycobacteria may induce pro-inflammatory response in macrophages upon internalization (Aleman et al., 2007; Krysko et al., 2006; Martin et al., 2014; Molloy et al., 1994). Neutrophils undergoing non-pathogen induced apoptotic cell death do not induce pro-inflammatory responses in macrophages. From our studies, we observed that neutrophils infected with these isolates neither induced pro-inflammatory cytokines, (except IL-8) nor, induced cell death. IL-8 may function as anti-apoptotic agent as it has been proposed by others (Dunican et al., 2000a; Dunican et al., 2000b) or it may just attract macrophages to the site of dead cells in order to

be able to scan for 'eat me' signal (Gardai et al., 2006; Ravichandran, 2011). These observations together point to the presumed concept that neutrophils act as 'Trojan horses' for virulent mycobacteria. In general, efficient killing of mycobacteria could allow neutrophils to prevent infection at an early stage. However, when killing is not efficient, infected neutrophils may transport pathogens to distant sites through circulation (Abadie et al., 2005; Eruslanov et al., 2005; Eum et al., 2010).

The ways by which mycobacteria alter the defense mechanisms of neutrophils still remain largely unknown. There are three ways known by which neutrophils can kill intracellular pathogens; production of ROS, release of various granular contents and finally, formation of NET, the *in vivo* function of the latter is yet to be confirmed (Borregaard, 2010; Brinkmann et al., 2004; Hager et al., 2010; Nathan, 2006; Segal, 2005). Based on our study, we propose a model of intracellular survival whereby these *M. avium* clinical isolates exploit the host defense responses and promote its own survival. Both these isolates prevent the activation of neutrophils that involves the p38 MAPK pathway. Inhibition of the release of granules into phagosomes promotes survival of the pathogens in spite of differential ROS induction. Limited activation of neutrophils prevents it from pathogen induced-apoptosis, thereby, allowing them to act as Trojan horses, ultimately, engulfed by the primary host, the macrophage.

3.8 Extracellular killing

Upon activation by pathogens or receptor ligands, neutrophils release granular contents into phagosomes and antimicrobial molecules of the granules contribute to the killing of intra phagosomal-pathogens. In addition to release into the phagosomes, these contents are secreted out of the cell, and it is thought that they may contribute to the extra cellular killing of pathogens. However, such extracellular killing ability of neutrophils in the context of mycobacteria still remains to be studied. We hypothesized that pathogen-specific stimulation of neutrophils may influence the contents of granular release, and thereby, their killing capacity of extracellular pathogens. It is known that neutrophils can be artificially stimulated

with a combination of a fungal soluble factor cytochalsin B (cytB) and formylated-peptide, fMLP to degranulate both azurophilic and specific granules (Bentwood and Henson, 1980; Mitchell et al., 2008; Standish and Weiser, 2009; Zarember et al., 2007).

We found that such an artificial stimulation of neutrophils resulted in enhanced release of both azurophilic and specific granules into the supernatant, but not secretory vesicles, as expected (Fig. 11A-C). Interestingly, we observed that this supernatant was able to inhibit the growth of both isolates in cell free-medium (Fig. 11D and E). The ability of granular contents in inhibiting mycobacterial growth in culture medium was reported earlier (Borelli et al., 1999; Halaas et al., 2010; Jena et al., 2012; Martineau et al., 2007; Sonawane et al., 2011). Martineau et al. (2007) showed that human neutrophil peptides 1-3, cathelicidin LL-37 and lipocalin 2 are capable of killing Mtb in culture medium. In addition, they reported that both cathelicidin LL-37 and lipocalin 2 inhibited growth of the organism, the latter in an iron-dependent manner. Jena et al. (2012) demonstrated that azurophilic components are able to kill mycobacteria in culture medium by disintegrating the bacterial membrane. However, how these antimicrobial molecules are regulated and secreted in neutrophils is not clearly known. More importantly, whether or not the secretion of these potential antimycobacterial molecules in infected neutrophils is affected by mycobacteria is not analyzed till this date. Although neutrophils activated by cytB/fMLP retain the degranulation mediated ability in controlling extracellular growth of SCH 228/215 (Fig. 11D and E), degranulation of neutrophils infected with these isolates is very much reduced due to the inhibition of neutrophils activation. Therefore, it is likely that the supernatant from infected neutrophils may not be able to inhibit the extra cellular growth of these isolates. As we expected, supernatants from SCH 228/215-infected neutrophils failed to inhibit extra cellular growth of these isolates (SCH 228/215) (Fig. 12A and B). Due to the differential activation of neutrophils by these two isolates, components of granules that are released in the supernatant may vary from each other and from that of cytB/fMLP-stimulated supernatant. This limited activation of neutrophils by these isolates may have enabled the cells to secrete only few or certain antimicrobial molecules into the extra cellular mileu. In this line, we could demonstrate that the contents of supernatant from neutrophils obtained upon stimulation with potent activators, cytB/fMLP, could actually inhibit the extra cellular growth of these

isolates, but not *S. aureus* (SA) (Fig. 12C and D). On the other hand, strikingly, SCH 228/215-stimulated supernatants showed differential but significant inhibition of SA growth (Fig. 12C and D), which correlated with differential activation of neutrophils by these isolates. Our data suggest that pathogen induced receptor-mediated activation of neutrophils is important not only for intra-phagosomal killing but also, for extracellular killing. In addition, our data suggest that the level of activation of neutrophils may play a role in dictating the composition of the granular contents that are released from neutrophils. This difference in the composition of granular contents may either be due to different levels of expression of proteins, or simply due to the presence or absence of certain proteins. It is interesting to notice that mycobacteria induced supernatant of neutrophils inhibited extra cellular growth of SA, suggesting these supernatants might be able to control a secondary infection by SA in an *in vivo* scenario. It has been reported that the oxidative killing mechanism of neutrophils is involved in killing of SA (Selva et al., 2009; Standish and Weiser, 2009). However, based on our extra cellular growth inhibition data, we suggest that non-oxidative killing mechanism of neutrophils involving release of granules may also play a role in SA killing. This has also been shown by other groups (Corbin et al., 2008; Hermann et al., 1990). Animal studies have shown that elastase and cathepsin G are important for the in vivo clearance of SA (Belaaouaj et al., 1998; Reeves et al., 2002). iTRAQ labeling and mass spec identification of proteins present in the supernatants from neutrophils, either stimulated with cytB/fMLP or infected with these mycobacteria, may shed light on how these supernatants vary in their functions. Such analysis would help us to identify the specific proteins that are responsible for inhibition of these clinical isolates and may open up avenues for use in therapeutic application in mycobacterial diseases.

3.9 Neutrophil-macrophage interaction

Neutrophils and macrophages are phagocytic cells that cooperate during inflammation and tissue repair, in addition to cell homeostasis (Silva, 2010a, b). Neutrophils provide the first line of defense against infection. However, their prolonged activation leads to tissue injury. Neutrophils undergo constitutive apoptosis and are engulfed by tissue macrophages or

monocytes in order to maintain neutrophil homeostasis (deCathelineau and Henson, 2003). During infection, pathogens may induce apoptosis in neutrophils. Clearance of apoptotic neutrophils can result in either suppression or stimulation of pro-inflammatory responses in engulfing macrophages, which may modulate macrophage activation and microbicidal activity (Lucas et al., 2003; Torchinsky et al., 2009; Zheng et al., 2004). Many studies have suggested that neutrophil-macrophage interaction may contribute to host defense against mycobacterial infection (Jena et al., 2012; Tan et al., 2006). Activation of both neutrophils and macrophages is important for this response. However, such investigation of neutrophil-macrophage interaction towards the control of mycobacterial growth was not yet done using human *M. avium* isolates. Our co-culture experiments demonstrated that the addition of naïve or LPS-stimulated neutrophils did not affect the intracellular survival of these human isolates in infected human macrophages (Fig. 13). This suggests that these isolates may prevent the interaction between neutrophils and macrophages thereby preventing the control of infection.

In addition to cytokines and chemokines, granular contents are released from neutrophils that contribute to the control of mycobacterial infection in macrophages (Soehnlein et al., 2009). Also, we have shown that granular contents from neutrophils are capable of inhibiting the growth of mycobacteria. However, the role of pathogen induced degranulation in the interaction between neutrophils and macrophages towards the control of mycobacteria is not investigated till this date. Here, using non-contact co-culture experiments, we showed that the addition of degranulated supernatant from either uninfected neutrophils or mycobacteria-infected neutrophils to infected macrophages did not affect the bacterial burden in the macrophages (Fig. 14). This may be due to the fact that the degranulation in infected neutrophils is inhibited by these mycobacterial isolates.

Neutrophils are attracted to the site of infection by infected macrophages. They need further activation from infected macrophages to exert their immune responses. It has been shown that contents from neutrophil granules are able to activate macrophages towards the control of mycobacterial infection (Ribeiro-Gomes et al., 2007; Soehnlein et al., 2009; Sonawane et al., 2011). However, whether infected macrophages influence the degranulation of infiltrated neutrophils is not studied so far. Our degranulation kinetics study showed that macrophages

infected with these isolates induced exocytosis of specific/gelatinase granules predominantly in naïve neutrophils and did not induce azurophilic and secretory granules (Fig. 15). These data suggest that mycobacteria infected macrophages prevent degranulation of azurophilic and secretory granules and induce specific/gelatinase granules in naïve unstimulated neutrophils. Exocytosis of secretory vesicles is required for the expression of receptors in the surface that are in turn required for the complete activation of neutrophils. Also, contents of azurophilic granules such as myeloperoxidase, defensins, neutrophil elastase, serine proteases etc., are shown to be involved in the activation of macrophages towards the control of mycobacteria. By preventing azurophilic and secretory granules release, these human isolates could possibly prevent activation of both unstimulated neutrophils and infected macrophages, thereby, sabotaging the outcome of the interaction between neutrophils and macrophages. Specific/gelatinase granules contain many tissue-degrading enzymes called matrix metalloproteinase (MMPs). The MMP family of enzymes contributes to both normal and pathological tissue remodeling. They have been shown to play a role in the migration of normal and malignant cells through the body. It was reported that in tuberculosis matrix metalloproteinase-1 is regulated by a p38 MAPK-dependent mechanism (Rand et al., 2009). In our study we observed predominant exocytosis of specific/gelatinase granules from naïve-uninfected neutrophils induced by SCH 228/215 *M. avium*-infected macrophages. This result suggests that degranulation of specific/gelatinase granules may lead to the further accumulation of immune cells including monocytes, T cells and neutrophils, which are necessary for the formation of granuloma around the infected macrophages.

3.10 Chemotaxis

Mycobacteria infected macrophages attract neutrophils to the site of infection by releasing cell mediators. The mechanisms by which neutrophils are attracted to the site of mycobacterial infection are not fully characterized. Lowe et al. (2012) have summarized the possible cell mediators that may be involved in neutrophil chemo attraction. We have previously shown that in addition to the well known cytokines and chemokines, infected macrophages release particles associated with HSP-70 that are chemoattractive to

neutrophils (Hartmann, 2006). In this study we have identified these particles as exosomes that are known to act as molecule carriers during immune cell-cell communication (Gutierrez-Vazquez et al., 2013). We showed that exosomes isolated from mycobacteria-infected THP macrophages attracted human neutrophils (Fig. 17). This data suggest that exosomes from mycobacteria-infected macrophages may be carriers of molecules that can attract neutrophils. Singh et al. (2012) demonstrated that in trans-well cell migration assay exosomes from Mtb H37Rv-infected macrophages could induce migration of macrophages, neutrophils and T cells from mouse splenocytes when compared to exosomes from uninfected macrophages. Furthermore, it has been reported that certain mycobacterial lipid components are released through exosomes and such pathogen-associated molecules may play a role in suppression of host immune response, probably by suppressing IFN- γ mediated activation of macrophages (Singh et al., 2011). However, one cannot rule out the possibility that the host factors present in or on the surface of the exosomes can also attract neutrophils, in addition to mycobacterial components. Analysis of our mass spec data from exosomes resulted in identifying putative proteins that may be involved in the chemoattraction of neutrophils (Table 2). In addition to the host proteins, we also observed that some bacterial proteins were present in those exosomes (Table 1). Since, proteins of these clinical strains were not characterized previously, we have restricted our similarity search to the genus, *Mycobacterium*. Since it is likely that the amount of mycobacterial antigens that might be present in exosomes from infected macrophages is lower than that present in mycobacteria itself, we therefore focused on the host factors that may be involved in the neutrophil chemoattraction.

3.11 Exosomes and their components as potential chemoattractants

The identification of the protein and lipid composition of exosomes may provide an insight into possible functions of these extracellular vesicles and how these molecules act in mycobacterial infection in granuloma. Here, as an example, we have focused on galectins due to the fact that it has been shown to be a potential chemotactic factor secreted via exosomes, that may be responsible for neutrophil chemotaxis (Gonzales et al., 2009;

Gonzalez-Begne et al., 2009; Koumangoye et al., 2011; Welton et al., 2010).

Galectins (Gal) are a family of proteins with the ability of binding to β-galactosides. To date, 15 members have been identified which differ in structure and in terms of the multivalent interactions they can establish with specific glycosidic structures. However, all galectins contain conserved carbohydrate-recognition domains (CRDs) of about 130 amino acids. In mammals, galectins are widely expressed by normal and pathological tissues. Due to their lack of signal sequence, galectins are thought to be secreted through a non-classical pathway (Hughes, 1999). In fact, secretion of galectins via the exosome pathway has been shown recently (Gonzales et al., 2009; Gonzalez-Begne et al., 2009; Koumangoye et al., 2011; Welton et al., 2010). Galectins also exist in the nucleus (Liu et al., 2002). Extracellularly, galectins can bind to cell-surface glycoconjugates that contain suitable galactose-containing oligosaccharides and glycoproteins in the extracellular matrices, such as laminin, fibronectin and elastin. Crosslinking of cell-surface glycoconjugates with galectins can trigger a cascade of transmembrane signaling events. Because of these binding abilities, galectins operate as readers of the glycocodes for leukocytes (Leffler et al., 2004). A diverse range of biological functions involved in immune and inflammatory responses and tumor development have been reported for galectins over the last decade, including roles in modulating cell–matrix interactions, trafficking, cytokine secretion, proliferation and survival, thus placing galectins into a growing list of important regulatory elements of immunity (Elola et al., 2007; Rabinovich et al., 2004; Yang et al., 2008)

Interestingly, we have found Gal-1, 3 and 9 to be present in the exosomes from infected macrophages (Table 1). Gal-1 and Gal-3 are probably the most ubiquitously expressed members of the galectin family. Gal-1 has been found to regulate multiple facets of adaptive and innate immune responses (Kuwabara et al., 2003; Norling et al., 2009; Rabinovich et al., 2002). Auvynet et al. (2013) showed that galectin-1 is a chemo attractant for neutrophils. Gal-1 is not exclusively chemotactic for neutrophils, as it has also been shown to influence the migration of dendritic cells and monocytes (Fulcher et al., 2009; Malik et al., 2009). It inhibits the release of inflammatory mediators from neutrophils (Rabinovich et al., 2000). Other galectins including Gal-3 and Gal-9 can also regulate the immune and inflammatory responses. Gal-3 has been shown to function as a chemoattractant for monocytes,

macrophages and neutrophils (Bhaumik et al., 2013; Nieminen et al., 2008; Sano et al., 2000). Similar to Gal-1 and Gal-3, Gal-9 can also induce T cell apoptosis (Fukumori et al., 2003). Gal-9 has been characterized a chemoattractant for monocytes, macrophages and eosinophils (Hirashima, 2000; Kuwabara et al., 2003; Nieminen et al., 2008). Given such functions attributed to Gal-1, 3 and 9, this makes galectins a very interesting candidate for our further studies regarding the function of exosomes as a potential chemoattractants.

In this study we used two clinical strains of *M. avium hominissuis*, SCH 228 and SCH 215 that have been previously characterized to be differential with regard to their generation time within macrophages as a feature of mycobacterial virulence. Such variation in virulence may be responsible for differences in disease outcome. We tried to understand the cell autonomous functions of neutrophils in mycobacterial infection and also how clinical *M. avium* strains considered of high and low virulence regulate such capacities of neutrophils in the evolutionary arms race. Our study demonstrates that SCH 228 and SCH 215 limit the activation of human neutrophils by differentially down regulating the activation of p38 MAPK pathway. Such differential regulations promote bacterial survival by impeding the p38 MAPK-mediated immune functions such as ROS, degranulation and cytokine induction in neutrophils. To the best of our knowledge, this is the first report on the human neutrophils' autonomous defense mechanisms and how human clinical *M. avium* strains affect them. The virulence based differential regulation of p38 MAPK mediated immune functions of human neutrophils may account for differences in the pathogenesis. Now it is becoming clear that in addition to host factors, phenotypic differences among different strains of mycobacteria are also involved in key aspects of pathogenesis. Moreover, this study underscores degranulation as a key function of neutrophils in limiting extracellular *M. avium* growth. As a future perspective, it is certainly worthwhile to do extensive comparative proteomic analyses of cytB/fMLP and *M. avium* stimulated supernatants from neutrophils in order to pin point possible factors that might be able to control *M. avium* infection. Finally, this study has also opened up new avenues to pursue certain unanswered questions regarding the functions of exosomes as potential chemoattractants as well as immune modulators in mycobacterial infection.

4 Summary

Neutrophils are phagocytes that are known to be fast responders and reach the site of an infection as an innate immune response. Neutrophils, in addition to monocytes and macrophages, have been implicated during the initial phase of mycobacterial infection. However, neutrophils are less well studied than other components of the host response to mycobacteria such as the macrophages, the natural primary host cell and T cells. Whether neutrophils are able to actively kill mycobacteria and thus contribute to mycobacterial clearance remains controversial. Our group has shown that human neutrophils are able to phagocytize and kill the avian type strain *Mycobacterium avium* TMC 724 (*M. avium*) *in vitro*. In this study we used two clinical strains of *M. avium hominissuis*, SCH 228 and SCH 215 that have been previously characterized to be differential with regard to their generation time within macrophages as a feature of mycobacterial virulence. Such variation in virulence may be responsible for differences in disease outcome. Here we tried to understand the cell autonomous functions of neutrophils in mycobacterial infection and also how clinical isolates of *M. avium hominissuis* (SCH 228 and SCH 215) considered of high and low virulence impact on neutrophil functional capacities. Our study demonstrates that SCH 228 and SCH 215 limit the activation of human neutrophils by differentially down regulating the activation of p38 MAPK pathway. Such differential regulations promote bacterial survival by impeding the p38 MAPK-mediated immune functions such as ROS, degranulation and cytokine induction in neutrophils. To best of our knowledge, this is the first report on how the virulence of clinical *M. avium hominissuis* isolates impacts on various innate immune functions of human neutrophils. We also demonstrated the ability of degranulation from cytB/fMLP-activated human neutrophils in controlling the extracellular growth of clinical *M. avium hominissuis* isolates, SCH 228 and SCH 215. Furthermore, the degranulated supernatants from neutrophils infected with these isolates show the capacity to inhibit extracellular growth of *Staphylococcus aureus* whereas they have no bactericidal effect on SCH 228 and SCH 215 themselves.

Investigation of neutrophil-macrophage interaction in the defense of these clinical *M. avium* isolates revealed that neither naïve nor LPS-stimulated neutrophils when co-cultured with infected human macrophages did impact on intra-macrophage bacterial survival of SCH 228 and SCH 215. However, macrophages infected with these isolates induce predominantly degranulation of specific/gelatinase granules in naïve neutrophils, indicating that neutrophil-macrophage interaction impacts on the microenvironment of local infection thereby potentially contributing to disease pathogenesis. Furthermore, this study underscores the importance of exosomes released from macrophages infected with *M. avium* as a cell-mediator molecule involved in the interaction of infected macrophages with other immune cells, as these exosomes attract human neutrophils. As a perspective, our finding that cytB/fMLP stimulated degranulation controls the extracellular growth of clinical *M. avium hominissuis* isolates, SCH 228 and SCH 215, encourages us to further carry out extensive comparative proteomic analyses of cytB/fMLP and *M. avium* stimulated supernatants from neutrophils in order to identify possible factors that might be able to control *M. avium* infection and may qualify as potential anti-mycobacterial drugs. Also, proteomic analysis of exosomes from infected macrophages suggests follow-up studies to identify the potential chemoattractive factors for neutrophils. This would allow one to study the role of such factor(s) present in the exosomes in the interaction of infected macrophages and neutrophils, and possibly, other immune cells.

5 Zusammenfassung

Mykobakterien stellen als humanpathogene Erreger weltweit eine Bedrohung dar. Allein *Mycobacterium tuberculosis (Mtb)* war im Jahr 2012 für 1.3 Millionen Tote und 8.6 Millionen Primär- bzw. reaktivierte Infektionen verantwortlich. Nicht tuberkulöse Mykobakterien (NTM), wie die Vertreter des *Mycobacterium avium* complex (MAC) sind opportunistische Keime bei immunsupprimierten Individuen. Seine größte Bedeutung erlangte *M. avium* im Rahmen der AIDS-Pandemie als lebensbedrohlicher Erreger bei HIV-infizierten Patienten. Nach einem, durch die Einführung der hochaktiven antiretroviralen Therapie (HAART) bedingten, Rückgang der Inzidenz Ende der 90er Jahre wird aktuell eine Renaissance von MAC Infektionen in der HIV-infizierten Population der westlichen Welt beobachtet. Darüber hinaus steigt die Inzidenz von MAC Infektionen auch bei immunkompetenten Patienten, insbesondere den Älteren, bei denen sich Infektionen der Lunge auf dem Boden einer pulmonalen Prädisposition manifestieren, die mit einer hohen Mortalität einhergehen.

M. avium ist ein fakultativ intrazellulärer Erreger, dem vorzugsweise Makrophagen und Monozyten als Wirtszellen dienen, der aber auch in nicht professionellen Phagozyten persistieren kann. Neutrophile Granulozyten sind die erste Linie der Verteidigung gegen mikrobielle Pathogene, dennoch wurde ihnen lange Zeit keine Bedeutung bei Infektionen mit intrazellulären Erregern zugeschrieben. Neuere Untersuchungen in Tiermodellen, in vitro Studien mit humanen Granulozyten sowie klinische Beobachtungen lieferten Hinweise darauf, dass Neutrophile einen direkten Beitrag zur Eliminierung von Mykobakterien leisten. Die Bedeutung dieses Beitrages und seine Einordnung in den Hintergrund der bisher bekannten Patho- und Immunmechanismen für Mykobakteriosen sind ungeklärt. In Untersuchungen, die dieser Arbeit vorausgingen, konnten wir zeigen, dass der aviäre Typstamm *Mycobacterium avium* TMC 724 (*M. avium*) durch neutrophile Granulozyten *in vitro* effektiv eliminiert wird. Virulente Mykobakterien können in Makrophagen einen phagosomalen Arrest verursachen, der ihre Degradierung im Phagolysosom verhindert. Somit können virulente Mykobakterien den Makrophagen als Wirtzelle nutzen und sich ungehindert replizieren. In dieser Studie haben wir zwei klinische Isolate von *M. avium*

hominissuis (SCH 228 und SCH 215) eingesetzt, die vormals basierend auf ihrer Generationszeit im Makrophagen als hoch und niedrig virulent charakterisiert wurden.

Derartige Virulenzunterschiede werden mutmaßlich für unterschiedliche Krankheitsverläufe verantwortlich gemacht. Wir haben untersucht, welchen Einfluss die unterschiedlich Virulenz dieser klinischen Isolate von *M. avium hominissuis* (SCH 228 and SCH 215) auf die funktionellen Eigenschaften von neutrophilen Granulozyten hat. Wir konnten zeigen, dass SCH 228 und SCH 215 nicht durch humane Neutrophile abgetötet werden, und dass beide Stämme den Prozess der Phagosomenreifung blockieren. Intrazelluläre Abtötung von Pathogenen durch Neutrophile und die Induktion der dafür notwendigen Prozesse, wie Degranulation und Freisetzung von ROS und Zytokinen ist unmittelbar mit einer Aktivierung des p38 MAPK Signalweges assoziiert. In Western Blot Analysen konnten wir zeigen, dass beide Isolate p38 MAPK herunterregulieren, wobei SCH 228 p38 MAPK für bis zu 60 min nahezu vollständig abschaltet, während SCH 215 eine moderatere Abschwächung der Aktivierung von p38 MAPK verursacht. Diese differentielle Induktion von p38 MAPK durch die beiden Isolate führt nach ihrer Phagozytose durch humane Neutrophile zu einem unterschiedlichen Aktivierungsmuster hinsichtlich Degranulation, ROS- und Zytokinfreisetzung. So induziert das niedrig virulente Isolat, SCH 215, 8-10 mal soviel ROS wie das hoch virulente Isolat SCH 228. Wir konnten ferner zeigen, dass die cytB/fMLP aktivierte Degranulation von humanen Neutrophilen einen bakteriostatischen Effekt auf das extrazelluläre Wachstum von beiden klinischen *M. avium hominissuis* Isolaten hat. Darüber hinaus haben die degranulierten Überstände von Neutrophilen nach Infektion mit SCH 228 und SCH 215 einen differentiellen bakteriziden Effekt auf *Staphylococcus aureus,* wohingegen sie das extrazelluläre Wachstum von SCH 228 und SCH 215 selbst nicht beeinflussen. Die Untersuchung der Interaktion von Makrophagen und Neutrophilen in Ko-Kultur Experimenten zeigte, dass weder naive noch LPS-stimulierte Neutrophile einen Einfluss auf das intrazelluläre Überleben von SCH 228 und SCH 215 im Makrophagen hatten. Allerdings induzierten *M. avium hominissuis* infizierte Makrophagen die Degranulation naiver neutrophiler Granulozyten mit einer Prädominanz von spezifischen/Gelatinase Granula. Dies impliziert, dass die Interaktion von Neutrophilen mit Makrophagen in der Immunabwehr von *M. avium hominissuis* einen Einfluss auf die lokale Mikro-Umgebung nimmt, der potentiell zur Pathogenese der Infektion beiträgt. Im Bezug

auf die Makrophagen-Neutrophilen-Interaktion unterstreichen unsere Ergebnisse die Bedeutung von Exosomen, die nach Infektion von Makrophagen mit *M. avium hominissuis* freigesetzt werden, als zelluläre Mediatoren, da diese Exosomen direkte Chemoattraktanten für Neutrophile sind. Die Tatsache, dass die cytB/fMLP induzierte Degranulation von Neutrophilen einen bakteriostatischen Effekt auf das extrazelluläre Überleben von klinischen *M. avium hominissuis* Isolaten hat, erlaubt als Ausblick dieses Projektes die vergleichende Proteom-Analyse von cytB/fMLP und *M. avium* stimulierten Überständen zur Identifizierung potentieller Faktoren, die für diesen bakteriostatischen Effekt verantwortlich zeichnen. Ferner kann die Proteom-Analyse der Exosomen von infizierten Makrophagen potentielle Chemoattraktanten für Neutrophile identifizieren, deren Rolle in der Interaktion von infizierten Makrophagen mit Neutrophilen und möglicherweise anderen Immunzellen in der Folge untersucht werden könnte.

6 Materials and methods

6.1 Materials

6.1.1 Instruments

Items	Company, Place
Autoclave	Fritz Gössner Gmbh, Hamburg
Blotting chamber Criterion	Bio-Rad, Munich
Centrifuge	
3 S-R	Heraeus, Hanau
J-6B	Beckman, USA
Multifuge 4KR	Heraeus, Hanau
Biofuge fresco, Tabletop	Heraeus, Hanau
Centrifuge rotors, max. 4600 rpm	Heraeus, Hanau
Constant cell disruption systems	IUL Instruments, Königswinter, Germany
Electrophoresis power supply, PowerPac-300	Bio-Rad, Munich
Fluorescent Activated Cell Sorter (FACS system) Calibur	BD Heidelberg
Freezer (-20°C) Liebherr Premium	Liebherr, Ochsenhausen
Freezer (-20°C) Hera Freeze, Kendro	Heraeus, Hanau
Fridge	Liebherr, Ochsenhausen
Geldoc2000	Bio-Rad, Munich
Ice machine	Ziegra, Isernhagen
Incubators	
CO2 incubators,	Thermo Scientific Forma Thermo, Langenselbold
Incubator with shaker,	Innova 4200 New Brunswick Scientific, USA
Laminar flow, LaminAir Holten,	Thermo Scientific Thermo, Langenselbold
Light Cycler PCR, Light cyler 480 SYBR Green I master	Roche diagnostics, Mannheim, Germany
Liquid nitrogen tank, Arpege 170	AirLiquide, Langenfeld
Magnetic stirrer, IKA, Combimag,	RCH Janke& Kunkel AG, Staufen
Microscope	
Light microscope (cell count),	Axiovert-25 Zeiss, Cologne
Micropipette plus 0.2-2 µL, 2-20 µL, 10-100 µL, 20-200 µL and 100-1000 µL	Thermo Lab Systems
Multipipette plus	eppendorf, Hamburg
Nano pure Diamond	Reinstwassersysteme Werner, Barnstedt
Orbital shaker, MR3000	Heidolph, Schwabach
pH-meter 766 Calimatic	Knick, Zweibrücken
Pipette boy,	Accujet Brand, Wertheim
Rotator, Heidolph Reax-2	Heidolph, Schwabach

Scales, Sartorius Laboratories	Sartorius, Heidelberg
Special accuracy weighing machine, Genius	Sartorius, Heidelberg
Stirred Ultrafiltration Cell (Amicon)	Millipore, Schwabach
Thermo mixer comfort, heating block	Eppendorf, Hamburg
Vacuum pump, Model RV3	Edwards, Kruetzwertheim
Vortex, Genie 2	Scientific Industries, USA
Water bath, GFL 1086	GFL, Burgwedel

6.1.2 Consumables

Items	Company, Place
15ml polypropylene test tubes, CellStar	Greiner Bio-One, Frickenhausen
50ml polypropylene test tubes, CellStar	Greiner Bio-One, Frickenhausen
Cannula (sterile) 26G,	Terumo Neolus, Belgium
20G, 27G	BD microlance3, Spain
Cell scraper (25 cm)	Sarstedt, Nümbrecht
Chemotaxis Plates (3 and 5 µm)	Neuro Probe, USA
Cotton swabs (Q-tips)	
Cover glasses for Haemocytometer Superior	Marienfeld Laboratory Glassware, Lauda-Königshofen
Cryo vials (sterile) 1.8 ml, CellStar	Greiner Bio-One, Frickenhausen
Eppendorf tubes, 0.5-2.0 ml	Sarstedt, Nümbrecht
FACS tubes	BD Falcon, Heidelberg
Filters (sterile), 0.45 and 0.2 µm	Nunc, Langenselbold
Microscope slides	Engelbrecht, Edermünde
Microtitre plates, 96 wells, non sterile	Nunc, Langenselbold
Neubauer chamber/Haemocytometer	Brand, Wertheim
Parafilm M	Brand, Wertheim
Pasteur pipette (230 mm)	Volac, Wertheim
Transfer pipettes (sterile) 5,10 and 25 ml	Sarstedt, Nümbrecht
Sealing tape	Dynex, Frankfurt
Syringes (sterile) 1, 5 and 10 ml	BD Falcon, Heidelberg
Syringes, Micro-fine (sterile), 1 ml, 29G, U-100	BD Falcon, Heidelberg
Steriflip, 0.2 µm pore size	Millipore, Schwalbach
Tissue culture plates 6-wells, 12-wells and 24-wells	Nunc, Langenselbold
96-well Fluorlumi white plates	Nunc, Langenselbold

Tissue culture flasks	Nunc, Denmark
Venofix safety	Braun Germany

6.1.3 Chemicals and reagents

Items	Company, Place
Acid citrate dextrose (ACD)	Sigma, Steinheim
Beta galactosidase	Sigma, Seelze, Germany
BSA (Bovine Serum Albumin fraction V)	Serva, Heidelberg
BBL Middlebrook ADC enrichment	BD, Heidelberg, Germany
C-12 FDG (5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside)-	Molecular Probes, Germany
Calcein AM (0.5 mg/ml DMSO)	Invitrogen, Spain
Complete protease inhibitor Cocktail tablet	Roche, Switzerland
Centrifuge tubes	Beckman Coulter, USA
Cytochalasin B	Sigma, Steinheim, Germany
Dextran-70 (Leuconostoc spp.)	Sigma, Steinheim, Germany
Difco Middlebrook/H9/7H10 broth	BD, Heidelberg, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Seelze, Germany
Dimethylformamide (DMF)	Sigma, Seelze, Germany
DMSO	Sigma, Steinheim
ECL plus Western blotting detection reagents	Amersham, Freiburg, Germany
Ethanol	Roth, Karlsruhe, Germany
EDTA (Ethylene diamine tetra acetic acid)	Sigma, Steinheim
FACSRinse, FACSFlow, FACSClean	BD Biosciences, Belgium
Ficoll	Amersham Bioscience, UK
Film developer Curix60	Agfa, Cologne
Fixer and Developer solutions	Agfa, Cologne
fMLP	Sigma, Steinheim
Glycerol	Roth, Karlsruhe, Germany
H2SO4 (96%)	Merck, Darmstadt
Hank's balanced salt solution (HBSS)	
Without Ca2+ and Mg2+	Biochrom AG, Berlin
With Ca2+ and Mg 2+	Biochrom AG, Berlin
HCL	Roth, Karlsruhe, Germany
Hepes, 1 M	Gibco, Karlsruhe
Histopaque-1077	Sigma, Steinheim
Hybond-P PVDF transfer membrane	Amersham, Freiburg, Germany

Hydrogen peroxide (H2O2)	Roth, Karlsruhe
Hyper film	Amersham, Freiburg, Germany
Interleukin-8 (IL-8)	R and D Systems, UK
L-Glutamine	Sigma, Steinheim
LPS, Ultrapure E. coli0111: B4 (TLR4 ligand)	Sigma, Seelze, Germany
Luminol (3-Aminophthalhydrazide)	Invitrogen, Germany
Lumiglo reagent	Cell signaling, Frankfurt
Phorbol myristate acetate	Sigma, Seelze, Germany
Pre-stained protein marker	Bio-Rad, Munich
Semi-dry transfer cell	Bio-Rad, Munich
SDS PAGE gels	Bio-Rad Munich
SDS PAGE running buffer	Bio-Rad, Munich
Transfer buffer	Bio-Rad, Munich
NaCl	Roth, Karlsruhe
Nitrocellulose membrane	Life Technologies
NuPAGE Bis-Tris mini gels	Life Technologies
P38 MAPK inhibitor	Sigma, Seelze, Germany
Penicillin/Streptomycin (10,000 U)	Biochrom AG, Berlin
Precision Plus Protein Standards Dual Color	Bio-Rad, Germany
Peroxidase, Horseradish	Calbiochem, Darmstadt, Germany
SiMAG-Octaecyl (C-18) size 1 µm	Chemicell, Berlin, Germany
Sodium azide (NaN3)(0.05%)	Fluka, Steinheim
Sodium Pyruvate, 100 mM	Gibco, Karlsruhe
Sterifix injection filter 0.2 µm injection	Braun Germany
Sucrose, Molecular biology grade	Calbiochem, Darmstadt, Germany
Super oxide dismutase (SOD)	Sigma, Seelze, Germany
Trypan blue	Sigma, Steinheim
Tween®20 (Poly oxyethylene sorbitanmonolaurate)	Sigma, Steinhein
XCell II Blot Module	Life technologies
XCell sure Lock Electrophoresis System	Life technologies
Zymogen	Sigma, Steinheim

6.1.4 Commercial kits used

Items	Company, Place
Apoptosis Detection Kit Annexin V PE conjugated	BD Pharmingen, Heidelberg
BCA test kit	Pierce, Rockfort, USA
Duo set ELISA kit	R and D systems, UK

IL-1β	R and D systems, UK
IL-1RA	R and D systems, UK
IL-6	R and D systems, UK
IL-8	R and D systems, UK
IL-10	R and D systems, UK
TNF-α	R and D systems, UK
Leukogate (CD45 FITC/CD14 PE)	BD, Heidelberg, Germany

6.1.5 Antibodies

For Western blotting	
Anti-phospho p38 MAPK (1:1000)	Cell signaling, Frankfurt
Anti-beta actin (1:3000)	Cell signaling, Frankfurt
Anti-HSP70 (1:200)	Santa Cruz, Heidelberg, Germany
Anti-MHC I (1:1000)	Abcam, UK
Anti-MHC II (1:500)	Abcam, UK
Anti-CD86 (1:1000)	Santa Cruz, Heidelberg, Germany
Anti-Rab5 (1:250)	Santa Cruz, Heidelberg, Germany
Anti-Mouse (HRP-conjugated) (1:5000)	Cell signaling, Frankfurt
Anti-Rabbit (HRP-conjugated) (1:5000)	Cell signaling, Frankfurt
For degranulation study	
Anti-CD35 (FITC)	BD Pharmingen, Heidelberg
Anti-CD63 (PE)	BD Pharmingen, Heidelberg
Anti-CD66b (Alexa 647-APC)	AbDSerotec, Puchheim, Germany

6.1.6 Buffers, media and solutions

All buffers and solutions were prepared or diluted with either Nano pure water, sterile aqua ad iniectabilia or PBS

Aqua ad iniectabilia	Delta Select, Pfullingen
Phosphate buffered saline-Dulbecco (PBS)	Biochrom AG, Berlin
VLE (very low endotoxin)-RPMI Roswell Park Memorial Institute (1640) medium	Biochrom AG, Berlin

Medium for mycobacteria

7H9 broth	Middlebrook 7H9 broth-4.7 g Tween 80 (20%)-2.5 ml Glycerol-2.5 ml Aqua dest. - 895.5 ml ADC-100 ml
7H10 agar plates	Middlebrook 7H10 agar-19 g Tween 80 (20%)-2.5 ml Glycerol-5 ml Aqua dest. – 900 ml ADC-100 ml

Cell culture

Fetal Calf Serum (FBS) LPS-free	Heat inactivated before use, 30 min at 56°C
THP-1 cell medium	RPMI 1640 Glutamine-2 mM HEPES-10 mM Sodium pyruvate-1 mM Streptomycin-100 μg/ml Penicillin-100 U/ml β-mercaptoethanol-0.05 mM FCS-10%
TC medium	RPMI medium FCS-10%
Freezing medium	FCS-90% DMSO-10%

Preparation of samples for Western blotting

Lysis buffer B	Tris (H 6.8)-62.5 mM SDS-3% β-mercaptoethanol-1.5 % Glycerol-8.5% Protease inhibitor cocktail tablet (Roche)

Western blotting

Bio-Rad XT sample buffer (4x) (purchased)	Tris-HCl (pH 8.5)-1 M SDS-8%

	Glycerol-40% EDTA-2 mM Coomassie blue Phenol red
Transfer buffer 1x	Transfer buffer (NuPAGE) (20x) -25 ml Methanol-50 ml Antioxidant-500 µL Aqua dest. – 500 ml
TBST buffer (Wash buffer)	Tris (pH 7.5)-1 M NaCl-150 mM Tween 20-0.1%
Antibody dilution and blocking buffer	5% Milk powder in TBST
Neutrophil isolation Erythrocyte lysis	NaCl (0.2 and 1.6%)
ROS assay 2x luminol mix	RPMI medium (w/o phenol red)-12.5 ml HRP (400 U/ml in sterile water)-200 µL Luminol (5 µM in DMSO)-200 µL
Determination of CFU	
2x lysis buffer	Tritonx100-1% SDS-0.2% in PBS (cold) sterilized for 5 min
WTA serial dilution buffer	Tween 80-1% BSA-1% in distilled water (sterilized)

6.1.7 Bacterial strains

Strain	Characteristics	Source
SCH228 *M. avium hominissuis*	Isolated from the bone marrow of an AIDS patient	Kindly provided by PD Dr Georg Plum, Institute for Medical Microbiology, Immunology and Hygiene, Uniklinik, Cologne
SCH215 *M. avium hominissuis*	Isolated from the lymph node a five year old boy, suffering from cervical lymphadenitis	Kindly provided by PD Dr Georg Plum, Institute for Medical Microbiology, Immunology and Hygiene, Uniklinik, Cologne
SA *Staphylococcus aureus*		Kindly provided by PD Dr Georg Plum, Institute for Medical Microbiology, Immunology and Hygiene, Uniklinik, Cologne

6.1.8 Cell lines

Cell line	Characteristics	Source
THP-1	Human monocytic cell line derived from an acute monocytic leukemia patient	ATCC® TIB-202TM (Tsuchiya et al., 1980)

6.1.9 Softwares

Item	Company, Place
Cell Quest pro (FACS-Analysis)	BD, Heidelberg, Germany
Endnote	Thomson Reuter
Graph Pad Prism	Prism
LightCycler 480 Software release 1.5.0 SP1 version 1.5.0.39 (Plate)	Roche Diagnostics, Mannheim, Germany
FACS FlowJo	

6.2 Methods

6.2.1 Culturing and freezing of THP cell line

THP-1 (ATCC® TIB-202TM) is a monocyte-like cell line derived from a one-year-old boy suffering from monocytic leukemia (Tsuchiya et al., 1980). These cells were grown as cell suspension in RPMI medium containing 2 mM L-Glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.05 mM 2-mercaptoethanol and 10% fetal calf serum (FCS) at 37°C with 5% CO2 in humid atmosphere. To establish the culture, 4 x 10^5 viable cells/ml cells were seeded in 20ml media allowed to grow for 2-3 days and were sub-cultured at a split ratio 1/4 to 1/5 when the density reaches 1 x 10^6 cells/ml. For cell stocks, the cell pellet was suspended in freezing medium containing 90% FCS and 10% DMSO. 1 ml cell suspension was aliquoted in cryo-vial and stored in liquid nitrogen.

6.2.2 Differentiation of THP-1 cells to macrophages

Monocytic THP-1 cells were differentiated to macrophages by PMA treatment. Floating monocytic cells were pelleted, suspended in RPMI medium containing 10% FCS and 100 nM PMA at a concentration of 10 x 106 cells/ml and allowed to differentiate for 1-2 days to become adherent macrophages.

6.2.3 Preparation of mycobacterial stock P0

Mycobacterial strains, SCH228 and 215 which were previously isolated from patients materials sent to the IMMIH of the University Cologne for diagnostic investigations, were kindly provided by PD Dr Georg Plum. In order to maintain the virulence nature and a lab stock of the strains, these mycobacteria were passaged through BALB/c mice. Mice were infected intravenously with 1x10^7 M. avium. After 6-8 weeks, mice were sacrificed, spleens and livers were isolated aseptically, their homogenates were aliquoted and frozen at -80°C. These organ homogenates were called passage, P0.

6.2.4 Preparation of mycobacterial stock P1

Mycobacteria from organ passage P0 were streaked with an inoculum loop on 7H10 plates supplemented with ADC and PANTA, and incubated for 7-10 days. A single colony was suspended in sterile PBS and streaked again on several 7H10 agar plates. After another 7-10 days of incubation, mycobacteria from all the plates were harvested, pooled in 7H9 broth, and washed once with PBS by centrifugation at 4600 xg for 10 min. Finally, the pellet suspended in PBS was singularized by passing through 20G needle and stored at -80°C as 100 µL aliquots. An aliquot was used to determine the number of viable mycobacteria present in this 100 µL volume by determining colony-forming unit.

6.2.5 Determination of CFU

Nine hundred µL of PBS was added to the 100 µL mycobacterial aliquot and this suspension was singularized through a 20G needle. Ten-fold serial dilutions were made in PBS and 20 µL of the diluted samples in triplicate were dropped on 7H10 agar. Colony forming units (CFUs) were counted after a time period of 7-14 days. CFU of mycobacterial stocks were calculated as CFU/ml.

6.2.6 Culturing of human monocyte derived macrophages (HMDM)

A buffy coat was obtained from a 500ml blood donation of the same day from the University clinic blood bank, University of Cologne. The buffy coat was diluted 1:1 with PBS and 25 ml of the diluted buffy coat was overlaid on 15 ml Histopaque gradient solution. This gradient was centrifuged at 1600rpm for 30min without break. After centrifugation, the thin interlayer between plasma and RBC, which contains white blood cells and platelets were carefully transferred into a fresh 50 ml tube. The isolated cells were then washed twice with RPMI by centrifuging at 900 rpm for 10 min. The total number of cells were counted using a Neubauer chamber and adjusted to a density of $2x10^7$ cells/ml with RPMI medium containing 5% FCS. These cells were plated on human serum opsonized 6-wells or 24-wells plates as required. The monocytes were allowed to adhere on to the base overnight at 37°C in a 5% CO2 atmosphere. After incubation cells were washed with pre-warmed RPMI

medium to remove the non-adherent cells. The adherent cells were allowed to differentiate into macrophages for another 5-6 days. Cells were renewed with RPMI medium containing 10% FCS (TC medium) every second day. The differentiated macrophages were used for further experiments.

6.2.7 Growth rate of *M. avium* (SCH228 and 215) in HMDM

Six-well plates were seeded with 1x106 monocytes isolated from buffy coat and allowed to differentiate into HMDM for 7 days. Macrophages were then infected with SCH 228 and 215 with a ratio 1:10 at 37°C. The required number of bacteria was calculated and diluted from the mycobacterial stock. After 2 h infection, cells were washed thoroughly with warm RPMI medium to remove extracellular bacteria, replenished with fresh warm TC medium and further incubated at 37°C. At different time points (2, 48 and 72 h) infected cells were lysed in lysis buffer for 10min and plated on 7H10 agar plates. 7H10 agar plates were incubated at 37°C for 7-10 days for enumerating colony forming units (CFU). The CFU count obtained after 2 h was considered as the time zero post infection. The generation time of the mycobacteria was calculated by this formula generation time (h) = (time y- time x) / 3.3LOG (CFUy /CFUx), where, CFU x is the number of bacteria at earlier time whereas, CFU y is the number of bacteria at later time.

6.2.8 Neutrophil isolation

Peripheral blood (20 ml) was collected in 50 ml Perfusor syringe filled with 18 ml Dextran 70/0.9% NaCl and 2 ml Citrate-Dextrose solution (ACD), mixed gently and allowed to stand at RT for RBC to settle. After 1 h of RBC sedimentation, the top clear leukocyte rich plasma was overlaid on 15 ml Ficoll in a 50 ml centrifuge tube using a Venofix safety. This gradient was centrifuged at 2000 rpm at RT for 20 min without break. After the centrifugation, everything was removed except the RBC and neutrophils pellet. RBC present in the pellet was lysed by adding first 8 ml of 0.2% NaCl followed by 1.6% NaCl solution, each for 20-30 sec and washed with HBSS buffer (without Ca^{2+} and Mg^{2+}) containing 0.1% chicken egg albumin by centrifuging at 1000rpm for 5 min with break. After another wash, cells were

counted using Haemocytometer. The purity of the isolated neutrophils by this method as assessed by FACS was over 90%.

6.2.9 Killing of Mycobacteria by human neutrophils

Bacterial survival in human neutrophils was determined as per Corleis et al. (2012). Neutrophils (2.5 x 10^5 cells) were added to each 1.5 ml centrifuge tube and infected with mycobacteria (rato 1:1). After 15 min of infection, cells were centrifuged at 300 xg for 10 min at RT. The neutrophil pellet was washed with TC medium by centrifugation. After another wash, neutrophils were re-suspended in 500 µL of the TC medium. Infected neutrophils were further incubated at 37°C. At different time points (1, 2 and 3 h), infected cells were lysed with 500 µL of 2x lysis buffer for 5 min. Neutrophil lysates were then serially diluted 10-fold in WTA serial dilution buffer, plated on sterile on 7H10 agar plates and incubated at 37°C for 7-10 days for enumerating colony forming units (CFU). The CFU count obtained after 15 min was considered as the time zero post infection.

6.2.10 Preparation of Zymosan-activated serum

Blood from healthy donor was collected in the serum collecting tube and serum was harvested by centrifuging at 3000 rpm for 5 min at RT. Zymosan was dissolved at 1mg/ml concentration in the serum and incubated at 37°C for 30 min before they were heat inactivated at 56°C for 30 min. After inactivation, samples were centrifuged at 300 xg for 10 min and then the clarified supernatant was collected, aliquoted and stored at -20°C.

6.2.11 Chemotaxis assay

Human neutrophils were isolated from peripheral blood from healthy donors using Dextran-Ficoll method. Neutrophils suspended in 2 ml HBSS (without Ca2+ and Mg2+) containing 0.1% chicken egg albumin were stained with the fluorescent dye Calcein AM at 3 µM concentration for 20 min at 37°C with gentle shaking and washed twice thereafter with HBSS buffer. After cell counting, cells were adjusted to a density of 1 x 10^7 cells/ml in

HBSS buffer (with Ca^{2+} and Mg^{2+}). Chemoattractant dilutions of different concentrations were prepared in HBSS buffer (with Ca^{2+} and Mg^{2+}) and then carefully, pipetted onto the bottom plate (29 µl/well). The filter (3 µm pore size) was gently stacked onto the plate before 25µl of cell were pipetted onto the filter. Zymosan activated serum (ZAS; 0.1 mg/ml) was used as positive and HBSS buffer as negative control. After 90min of incubation, unmigrated neutrophils were removed carefully from the top of the filter and the filter was dried gently with Q-tips. Migration of the fluorescent neutrophils from filter to bottom plate was quantified by measuring the total fluorescence from plate as well as filter in Tecan. Each concentration was pipetted at least in triplicates and the migration assay was performed at least three times.

6.2.12 Coating of mycobacteria with C12-FDG

Coating of mycobacteria was performed according to Robinson et al. (2008). An aliquot of mycobacteria (in some experiments, mycobacteria were heat inactivated at 95°C for 20 min) washed with PBS by centrifuging at 12000 xg for 5 min at RT. Mycobacteria pellet was suspended in 190 µL of 100 mM NaHCO3 buffer (pH 9.6), mixed with 10µL of C12-FDG (stock 20 mM) and incubated in sonicator water bath at 37°C for 1 h with intermittent sonication for 10 sec every 10 min. After 1h incubation, 800 µL NaHCO3 buffer was added and mycobacteria were washed twice by centrifugation. Finally, bacteria were suspended in 200 µL NaHCO3 buffer. The coating was visualized by incubating with 5-10 µL of β-galactosidase for 10 min at 37°C under UV trans-illuminator.

6.2.13 Coating of magnetic beads with C12-FDG

Coating of magnetic beads was done according to Robinson et al. (2008). An aliquot of magnetic beads (15 µL) was suspended in 190 µL of 100 mM NaHCO3 buffer (pH 9.6), mixed with 10 µL of C12-FDG (stock 20 mM) and incubated in sonicator water bath at 37°C for 1h with intermittent sonication for 10sec every 10 min. After 1 h incubation, 800 µL $NaHCO_3$ buffer was added and beads were washed twice on magnet. Finally, beads were suspended in 200 µL $NaHCO_3$ buffer.

6.2.14 Phagosomal maturation study in human neutrophils

Human neutrophils were isolated from peripheral blood from healthy donors using Dextran-Ficoll method. Neutrophils were suspended in TC medium at a density $2.5x10^5$ cells well in 24-wells plate and infected with C12-FDG coated mycobacteria at MOI 10 for 15 min at 37C. C12-FDG coated magnetic beads were used as positive control for this assay. An aliquot of beads was added to cells for 10min before they were washed. After infection, neutrophils were harvested, pelleted at 300 xg for 10 min to remove extra cellular bacteria and re-suspended in 250 µL of TC medium before seeding in 24-wells plate. At different time points (0.5, 1, 2, 3, 4 and 5 h), cells were harvested and fluorescence of C12-FDG was measured using FACS. After gating the neutrophil population using Leukogate antibody in forward and sideward scatter, cells were analyzed for the fluorescence by FACScan using FlowJo software. The results were expressed as histogram for the fluorescence-intensity of cells in a population. Experiments were performed a minimum of three times in an independent manner.

6.2.15 Phagosomal maturation study in human macrophages

Twenty four-well plates were seeded with $2.5x10^5$ monocytes isolated from buffy coat and allowed to differentiate into HMDM for 7 days. Macrophages were then infected with C12-FDG coated mycobacteria at ratio 1:10 for 1 h at 37°C. C12-FDG coated magnetic beads were used as positive control for this assay. An aliquot of beads was added to cells for 10 min before they were washed. At different time points (0.5, 1, 2, 3, 4 and 5 h), cells were harvested and fluorescence of C12-FDG was measured using FACS. After gating the macrophage population using Leukogate antibody in forward and sideward scatter, cells were analyzed for the fluorescence by FACScan using FlowJo software. The results were expressed as histogram for the fluorescence-intensity of cells in a population. Experiments were performed at least two times in an independent manner.

6.2.16 Lysis of samples for Western blotting

Neutrophils were lysed and samples for western blotting were prepared as mentioned by al-Shami et al. (1997). To the pelleted neutrophils, boiling lysis buffer B was added, mixed and boiled for 7 min. Following the boiling, samples were snap cooled on ice and clear lysate was collected after centrifuging at 12000 xg for 10 min.

6.2.17 Western Blot Analysis

Neutrophils were washed with PBS and pelleted by centrifugation. Pellets were lysed for whole cell extraction in lysis buffer B plus inhibitors. Lysates were centrifuged, the supernatant was collected and used for protein determination prior to protein sample separation on a SDS-PAGE. Protein samples were prepared by mixing with loading dye and heating at 95°C for 5 min. Equal amount of protein was loaded on to the SDS gel consisting of a 12% resolving gel and a 5% stacking gel. The proteins were electrophoresed in a XCell sure Lock Mini-Cell Electrophoresis System and XCell II Blot Module from Invitrogen for 70-90 min at 120 V. Hybond-PVDF membrane was pre-wetted in 100% methanol for 5s followed by in water for 5min and then equilibrated in transfer buffer for 10 min. Care was taken not to allow the membrane to dry. The SDS gel was also equilibrated in transfer buffer for 10 min. A fibre pad was wetted in transfer buffer and placed on the transfer chamber. Over the pad two Whatman3 paper sheets of the size of the gel dipped in transfer buffer was placed. The gel was placed upon the stack and the membrane over the gel. The membrane and gel were sandwiched with another 2 sheets of whatman3 paper wetted with transfer buffer. After closing the chamber, transfer was done at 100 V for 90 min at 4°C.

After the transfer, the membrane was washed with wash buffer and blocked by incubating in blocking buffer for 1 h at 37°C on an orbital shaker. Then the membrane was washed three times (5 min each wash) with wash buffer at RT. Following the washing step, membrane was incubated for over night at 4°C on an orbital shaker with primary antibody diluted appropriately in antibody dilution buffer. After three washings, the HRP-linked secondary antibody was diluted appropriately in antibody dilution buffer and added to the membrane and incubated for 1 h at RT with constant shaking. Then, the membrane was washed three

times (5 min each wash) in wash buffer at RT. Membrane was placed on a polythene sheet and allowed to react with 1x Lumiglo reagent for 2-5 min. Finally, the membrane was exposed to an X-ray film (Hyperfilm) for 5-10 min in a hypercassette in the dark and developed in Western Blot Developer machine Curix 60.

For re-probing the membrane with different antibody, the membrane was stripped with stripping buffer at RT for 30 min. After incubation, membrane was washed twice with PBS and once with wash buffer, each 5 min. The membrane was blocked in blocking buffer for 1 h at RT before probing with different antibody.

6.2.18 Degranulation study

Cell surface expression of CD35, CD63, and CD66b was monitored for assessing degranulation of secretory, azurophilic, and specific granules, respectively, by flow cytometry (Simard et al., 2010). Heparized blood (5 ml) was overlaid on 5 ml of Histopaque 1077 and allowed to stand for 45 min at RT. Leucocyte rich supernatant (LRS) from the top was transferred to a tube containing 2 ml of HBSS/Hepes buffer (10 mM; pH 7.4). Following the count, cell density was adjusted to $5x10^6$ cells/ml concentration (Hartmann et al., 2001). For some experiments, human neutrophils were isolated from peripheral blood from healthy donors using Dextran-Ficoll method. Cells were incubated with fMLP (10µM) or mycobacteria (ratio 1:10) for 5 or 15 min at 37C. Following incubation, cells were pelleted at 300 xg for 10 min at RT and suspended in 100 µL of HBSS/Hepes buffer. Cells were stained with CD35 FITC (5 µL), CD63 PE (5 µL), CD66b Alexa 647 (APC) (2.5 µL), Leukogate CD45/CD14 (5 µL) and 7-AAD (5 µL). After staining, cells were washed once with HBSS/Hepes buffer. After gating the neutrophil population using Leukogate antibody in forward and sideward scatter, cells were analyzed for expression of various granule specific markers on cell surface by FACScan using FlowJo software. These results were expressed as histogram for the fluorescence-intensity of the cells in the gated population. Experiments were performed at least time times. For some other experiments, cells were pretreated at 37°C with p38 MAPK inhibitor (2 µM) for 30 min or IL-8 (50 pg/ml) for 15 min before they were infected.

To test the effect of cytB/fMLP on degranulation, neutrophils ($1x10^7$ cells/ml) suspended in RPMI medium supplemented with Hepes (25 mM) were first incubated with cytB (5 µg/ml) at 37°C for 5 min followed by 30 min incubation with fMLP (0.1 µM). Unstimulated neutrophils incubated for the same time at 37°C was used as the negative control for this experiment. To subtract the effect of solvents, corresponding volume of the solvent DMSO and DMF was added to the neutrophils and incubated, respectively. Following the incubation, cells were pelleted at 300 xg for 10 min at RT and suspended in 500 µL of HBSS/Hepes buffer. An aliquot of the cell suspension was stained with CD35 FITC (5 µL), CD63 PE (5 µL), CD66b Alexa 647 (APC) (2.5 µL), Leukogate CD45/CD14 (5 µL) and 7-AAD (5 µL). After staining, the cells were washed once with the HBSS/Hepes buffer. After gating neutrophil population using Leukogate antibody in forward and sideward scatter, cells were analyzed for expression of various granule specific markers on cell surface by FACScan using FlowJo software.

To study the effect of mycobacteria-infected macrophages on degranulation, macrophages ($2.5x10^5$ cells per well) in 24-wells plate were infected with mycobacteria (ratio 1:10) for 1 h at 37°C. After the infection, extra cellular mycobacteria were washed off and freshly isolated neutrophils were incubated with either uninfected macrophages or infected macrophages in 1:1 ratio. At different incubation times (30, 60 and 120 min), neutrophils were pelleted at 300 xg for 10 min at RT and suspended in 100 µL of HBSS/Hepes buffer. Cells were stained and analyzed for expression of various granule specific markers on cell surface by FACScan using FlowJo software.

6.2.19 Apoptosis assay

To assess the cell death of mycobacteria infected neutrophils, cells were incubated at 1 x 10^6 cells/ml in TC medium with mycobacteria (ratio 1:10) at 37°C for 15 min. After infection, neutrophils were pelleted at 300 xg for 10 min at RT, washed, re-suspended in 500 µl of TC medium and incubated at 37°C. At different time points (1, 2, 3, 4 and 5 h) cells were pelleted, suspended in binding buffer and stained with Annexin V-PE (AV) and 7-AAD for 10 min at RT, according to the manufacturer's instructions. Cells were washed and acquired

by FACS. After gating viable neutrophil population using Leukogate antibody in forward and sideward scatter, cells were analyzed for the Annexin positive cells in the gated population by FACScan using FlowJo software. These results were expressed as quadrant for the fluorescence-intensity of the cells in the gated population. AV positive cells were considered as apoptotic cells and AV + and 7-AAD + cells were considered as late apoptotic/necrotic cells.

6.2.20 ROS assay

Human neutrophils were isolated from peripheral blood from healthy donors using Dextran-Ficoll method and the cell density was adjusted to $2x10^6$ cells/ml concentration in ROS medium. Neutrophils (50 µL) were added to each well in 96-well fluorescence/luminescence white plate (Nunc). A hundred µL of luminol mix (2x) was added to each well. ROS medium (50 µL) or mycobacteria (ratio 1:50; 50 µL) or fMLP (2 µM; 50 µL) was added and immediately measured at Tecan using the program 'Senthil luminol 72 cycles' (72 cycles with 5 min interval and 100msec integration time). For ROS measurement for the co-infection experiment, neutrophils were infected alone with mycobacteria (SCH 228 and 215 (ratio 1:50 and 100), respectively; 50 µL) or together with both mycobacteria. For some experiments, an aliquot of p38 MAPK inhibitor (50 µL) was added to the row labeled 'p38 MAPK inhibitor' and incubated at 37°C for 30 min before they were infected with mycobacteria.

6.2.21 Cytokine measurements

Human neutrophils were isolated from peripheral blood from healthy donors using Dextran-Ficoll method and the cell density was adjusted to $2x10^7$ cells/ml concentration in HBSS buffer (with Ca2+ and Mg2+). Equal volume of cells and mycobacteria (ratio 1:50) were added into a 1.5 ml micro-centrifuge tube and incubated in a thermo mixer at 37°C with a gentle shaking. At different time points (1.5, 2, 4 and 6 h), cells were pelleted and the supernatant was transferred to another tube. This supernatant was further centrifuged at 12000 xg for 10 min at 4°C. The clarified supernatant was then transferred to another fresh

tube and frozen at -20°C. The levels of IL-6, 8, 10, IL-1β, TNF-α and IL-1ra present in the supernatants of neutrophils were measured using commercially available ELISA kits, according to the manufacturer's instructions. For some experiments, neutrophils were pretreated with p38 MAPK inhibitor (2 μM) or fMLP (10 μM) for 30 min at 37°C before they were infected or they were infected in the presence of SOD (20 μg/ml) or ROS (H_2O_2; 400 nM).

6.2.22 In vitro anti-bacterial assay

Neutrophils ($1x10^7$ cells/ml) suspended in RPMI medium supplemented with Hepes (25 mM) were first incubated with cytB (5 μg/ml) at 37°C for 5 min followed by 30 min incubation with fMLP (0.1 μM) (Standish and Weiser, 2009). Unstimulated neutrophils incubated for the same time at 37°C was used as the negative control for this experiment. To subtract the effect of solvents, corresponding volume of the solvent DMSO and DMF was added to the neutrophils and incubated, respectively. Neutrophils were infected with SA at ratio 1:1 or mycobacteria at ratio 1:10 for 30 min at 37°C. Following incubation, cells were pelleted at 300xg for 10 min at RT, the supernatant was transferred to another tube and centrifuged at 12000 xg for 10 min at RT. The clarified supernatant (100 μL) was added to a well in 24-well plate that contained 100 μL of mycobacteria ($1x10^6$ CFU/ml) in AMA buffer and incubated for 48 h. After incubation, 800 μL of WTA buffer was added serially diluted 10-fold in WTA serial dilution buffer and 20 μL of the diluted samples in triplicate were dropped on 7H10 agar. Colony forming units (CFUs) were counted after a time period of 7-14 days. For assessing the survival of SA, the clarified supernatant (100 μL) was added to 100 μL of SA (1x105 CFU/ml) and number of bacteria was enumerated after 2 and 4 h by CFU method.

6.2.23 Co-culture experiment

Macrophages ($2.5x10^7$ cells per well) in 24-well plate were infected with mycobacteria at MOI 10 for 1 h at 37°C. After infection, extra cellular mycobacteria were washed off, infected macrophages were incubated in 1:1 ratio with naïve/unstimulated or LPS-stimulated

or infected neutrophils. At 48 and 72 h, after washing off neutrophils, infected macrophages were lysed and the number of bacteria was enumerated after 2 and 4 h by CFU method. The number of bacteria enumerated from infected macrophages without neutrophils was used as control. In some other experiments, infected macrophages were incubated with degranulated supernatant from mycobacteria infected neutrophils and CFU was determined after 48 and 72 h of incubation.

6.2.24 Depletion of exosomes from FCS

Fetal calf serum (FCS) was thawed and heat inactivated at 56°C for 30 min. Then, FCS was centrifuged at 100,000 xg for 16 h at 4°C (Bhatnagar and Schorey, 2007). After centrifugation, the supernatant was pooled, sterile-filtered using 0.2 μm filter and stored at -20°C.

6.2.25 Exosomes isolation

Macrophages were infected and exosomes were isolated according to Bhatnagar and Schorey (2007) with slight modifications. THP macrophages, cultured in TC medium supplemented with 10% exosomes-depleted FCS, were infected with mycobacteria with a ratio 1:40 for 4 h and followed by extensive washing to remove extracellular bacteria. As control, macrophages were left uninfected. After 72 h, the TC medium was collected and centrifuged twice at 300 xg, 10 min to remove whole cells, followed by centrifugation at 1,200 xg for 10 min to remove any bacilli. The supernatant was centrifuged at 10,000 xg for 30 min. Then, the supernatant was centrifuged at 100,000 xg for 1 h at 4°C. The resulting pellet (exosomes) was suspended in 2.5 ml of 2.5 M sucrose/HBSS buffer and placed in ultra thin centrifuge tube. A dis-continuous sucrose gradient (6 ml of 2 M and 3 ml 0.25 M sucrose/HBSS buffer) was layered on top of the exosomes suspension. The gradient was then centrifuged at 100,000 xg for 16h at 4C. Gradient fractions (7x1 ml) were collected from the top of the gradient and fractions 3-5 were pooled, diluted with HBSS buffer, centrifuged at 100,000 xg for 1 h at 4°C. The resulting exosomes pellet was re-suspended in HBSS buffer and the protein concentration was measured using BCA method as per manufacturer's instructions.

6.2.26 Mass spectrometry

Digestion

Samples normalised to 70 µg protein in 20 µl were sonicated three times for 10 s. 2 µl 100 mM DTT were added and the samples were boiled to 96 °C for 10 min. After cooling down to room temperature, cysteines were alkylated with 2 µl 250 mM iodine acetamide. Samples were stored for 30 min in the dark at room temperature. Excess iodine acetamide was quenched with 0.5 µl 100 mM DTT. 200 µl 8M Urea with 0.1 M TEABC were added and the samples were transferred to Amicon Ultra™ centrifugal filter units (0.5 ml, 10K). Filter assisted sample preparation (FASP) with sequential Lys C (0.5 µg) and trypsin (1.75 µg) digestion was performed according to (3) with minor modifications: Briefly, 0.1 M TrisHCl was replaced by 0.1 M triethylammonium bicarbonate pH 8.5 (TEABC) in all buffers used. After digestion the samples were centrifuged and filtrates were collected. 150 µl 5 % acetonitrile in water were added to the filter, mixed with the residual sample and the units were centrifuged once more. C18 SPE cartridges (Strata-C18, 1 ml, Phenomenex) were activated with Methanol, washed with 80 % acetonitrile and equilibrated to 5 % acetonitrile in 0.1 M TEABC. Combined filtrates were applied, the cartridge were washed two times with 300 µl 5 % acetonitrile in 0.1 M TEABC and the peptides were eluted with 300 µl 80 % acetonitrile in 0.1 M TEABC. One third (100 µl) of the eluates were dried in a vacuum centrifuge and redissolved in 10 µl 0.5 M TEABC for ten minutes at room temperature

iTRAQ labelling

All reagents were used at room temperature. 70 µl Ethanol were added to the iTRAQ reagents (AB Sciex) and 25 µl were used for each sample containing approximately 6 µg peptides in 10 µl 0.5 M TEABC pH 8.5. After 2 h in the dark the reaction was quenched with 50 µl water and the labelled samples were combined. Volumes were reduced to approximately 20 µl in a centrifugal evaporator and 200 µl ultrapure were added. STRATA C18 cartridges were used for sample clean up as described above. However, this time, TEABC was replaced by 0.1 % formic acid in all steps. Desalted peptides were dried in a centrifugal evaporator and resuspended in 40 µl of ACN in 0.1 % formic acid.

SCX HPLC

Strong Cation Exchange (SCX) Chromatography was performed on a Biobasic SCX column (1 mm x 150 mm, Thermo) installed to an Ettan micro LC system (GE Healthcare) operated at a flow rate of 50 µl/min and 214 nm fixed wavelength detection. Buffer A was 25 % acetonitrile in 0.1 % formic acid adjusted to pH 3.5 with NaOH. Buffer B was 0.5 M NaCl in buffer A. Samples redisolved in 40 µl of 40 % acetonitrile in 0.1 % formic acid were injected onto the column equilibrated in buffer A. Peptides were separated using a gradient of 0 % B for 1.5 min, 0 % - 10 % B in 4 min and 10% - 80 % B in 8 min. Fractions were collected every 60 sec. 6 fractions were dried in a vacuum centrifuge, redissolved in 50 µl 5 % acetonitrile in 0.1 % TFA and used for subsequent LC-MALDI analysis.

MALDI spotting

Reversed phase nanoHPLC of SCX fractionated peptides was carried out on an Eksigent nanoLC 1D plus system (Axel Semrau GmbH, Sprockhövel, Germany) using a vented column setup comprising a 0.1-mm-by-20-mm trapping column and a 0.075-by-200-mm analytical column, both packed with ReproSil-Pur C18-AQ, 5 µm (Dr. Maisch, Ammerbuch, Germany) and operated at 40 °C. 18 µl sample were aspirated into the sample loop and a total of 30 ul was loaded onto the trap column using a flow rate of 6 µl/min. Loading pump buffer was 0.1 % TFA. Peptides were eluted with a gradient of 5 % to 35% acetonitrile in 0.1% TFA over 70 min and a column flow rate of 300 nl/min. 0.7 mg/ml alpha-Cyano-4-hydroxycinnamic acid (HCCA) in 95 % acetonitrile in 0.1 % TFA, 1 mM ammonium phosphate were fed in using a syringe pump operated at 150 µl/h and a post column T-union. 384 fractions (10 seconds) were deposited onto a MTB AnchorChip 384-800™ MALDI target (Bruker Daltonics, Bremen, Germany) using Eksigent MALDI spotter (Axel Semrau GmbH, Sprockhövel, Germany).

LC-MALDI MS/MS analysis

MALDI MS and MS/MS analysis were carried out on an Ultraflextreme™ MALDI Tof Tof mass spectrometer (Bruker Daltonics) operated with a laser repetition rate of 1 Ghz. The

process of data acquisition was controlled by Flexcontrol 3.0 and WarpLC. MALDI MS spectra were acquired over a mass range from 700 Da – 4000 Da. Spectra were calibrated externally using the Peptide Calibration Standard II (all Bruker Daltonics) on the designated target calibration spots. The laser was used with fixed energy setting and 3000 shots/spectrum were collected from random raster points. Precursor ions with signal to noise ratios equal or better than 10 were selected for MS/MS analysis. Identical peaks in adjacent spots were measured only once, preferentially from the spot with maximum peak intensity. The maximum number of MS/MS spectra per spot was limited to 20. However, this value was exceeded if no alternative positions had been available. Peaks appearing on more than 40 % of all spots were ignored. MS/MS spectra (3500 shots) were acquired with the instrument calibration and iTRAQ reporter ions as well as peptide immonium ions were used for internal recalibration.

Database searches and analysis of identified proteins

Daphnia Pulex (Tax ID 6669) entries (32413 sequences, 10481177 residues) were extracted from the NCBInr (Release of February 2. 2014). A composite database was generated with the Perl script "makeDecoyDB" (Bruker Daltoniks, Bremen, Germany) which added a shuffled sequence and a tagged accession number for each entry. The tagged decoy entries were used for the calculation of false positive rates in Proteinscape 3.0. Searches were submitted via Proteinscape (Bruker Daltoniks, Bremen, Germany) to MASCOT 2.2 (Matrix Science) and the following parameter settings were used for database searches: enzyme "trypsin" with 1 missed cleavage; fixed modifications "carbamidomethyl" ,"iTRAQ4plex (K)", "iTRAQ4plex (N-term)"; optional modifications "Methionine oxidation", "iTRAQ4plex (Y)", "Gln->pyro-Glu (N-term Q)". The mass tolerance was set to 15 ppm Da for MS and 0.8 Da for MS/MS spectra. Protein lists were compiled in Proteinscape. Peptide hits were accepted when the ion score exceeded a value of 25. Protein hits required at least one peptide hit exceeding a peptide score of 40. Using this set of search parameters, the false positive rate was below 0.5 % on the protein level.

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8 Abbreviations

°C	Degree Celsius
Ab	Antibody
ADC	Albumin-dextrose-catalase
Alexa 647	A photo-stable fluorescent conjugate
APC	Allophycocyanin
Aqua dest.	Distilled water
BCG	Bacille Calmette-Guérin
BM	Bone marrow
BMDM	Bone marrow derived macrophage
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming unit
Ctrl	Control
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol,
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gram
g	Gravitational acceleration
H2O	Water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h	Hour
HRP	Horseradish peroxidase
IFN- γ	Interferon gamma
IL	Interleukin
inf.	Infected
kDa	Kilo Dalton
Kg	Kilogram
L	Liter
LPS	Lipopolysaccharide
M. avium	*Mycobacterium avium*
M. tuberculosis; *Mtb*	*Mycobacterium tuberculosis*
mAb	Monoclonal antibody
MAC	Mycobacterium avium complex

MgCl2	Magnesium chloride
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
MOI	Multiplicity of infection
MΦ	Macrophage
n	Number of tests
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nano gram
ns	Not significant
ns	Non-stimulated
NTM	Non-tuberculous mycobacteria
OD	Optical density
P/S	Penicillin / Streptomycin
PANTA	Polymyxin b, Ampicillin, Nalidixic acid sodium salt, Trimethoprim, Amphotericin b
PBS	Phosphate buffered saline
PE	Phycoerythrin
pH	defines the acidity or basicity of an aqueous solution
HRP	Horse radish peroxidase
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RT-PCR	Real time PCR
s	Second
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SSC	Sideward scatter
TBST	Tris-buffered saline plus Tween-20
TMB	3,3′, 5,5′-Tetramethylbenzidine
TNFα	Tumor necrosis factor α
Tris	Tris (hydroxymethyl) amino methane
Tween	Polyoxethylene-sorbitan-monolaureate
U	Unit
ui	Non-infected
v/v	Volume per volume
w/v	Weight per volume
WB	Western blot
β-Me	β-mercaptoethanol
µl/µL	Microliter

9 Declaration

I hereby declare that the work in this thesis is original and has been carried out by me at the Internal medicine 1 of University Hospital and Institute for Medical Microbiology, Immunology und Hygiene, University of Cologne, under the supervision of Priv. Doz. Dr. med. Pia Hartmann and in partial fulfillment of the requirements of the Doctor of Philosophy degree of the University of Cologne. I further declare that this work has not been the basis for the awarding of any degree, diploma, fellowship, associateship or similar title of any university or institution.

Senthil Kumar Subramanian

May 2014

10 Acknowledgment

Gratitude is one of the least articulate of the emotions, especially when it is deep.

-Felix Frankfurter

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11 Curriculum vitae

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