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Original Publication:

Alexander Persson, Robert Blomgran, Daniel Eklund, Charlotte Lundstrom and Olle Stendahl, Induction of apoptosis in human neutrophils by Mycobacterium tuberculosis is dependent on mature bacterial lipoproteins, 2009, MICROBIAL PATHOGENESIS, (47), 3, 143-150.

<http://dx.doi.org/10.1016/j.micpath.2009.05.006>

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Induction of apoptosis in human neutrophils by *Mycobacterium tuberculosis* is dependent on mature bacterial lipoproteins

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Abstract

Modulation of immune cell apoptosis is a key evasion strategy utilized by *Mycobacterium tuberculosis* (Mtb). To be able to multiply within macrophages, the bacterium delays apoptosis and down-regulates pro-inflammatory activation in these cells, whereas apoptosis is rapidly induced in the potentially bactericidal neutrophils. Initial host-pathogen interactions between neutrophils and Mtb, subsequently leading to apoptosis, need to be investigated to understand the early features during Mtb infections. Opsonized Mtb were readily phagocytosed, and the immuno-mediated phagocytosis triggered early activation of anti-apoptotic Akt in the neutrophils but the bacteria still induced apoptosis to the same extent as non-phagocytosed Mtb. Mtb-induced apoptosis was strictly dependent on NADPH oxidase-generated reactive oxygen species, compounds shown to damage lysosomal granules. Despite this, we found no involvement of damaged azurophilic granules in Mtb-induced apoptosis in human neutrophils. Instead, the Mtb-induced apoptosis was p38 MAPK dependent and induced through the mitochondrial pathway. Moreover, Mtb deficient of mature lipoproteins lacked the determinants required for induction of neutrophil apoptosis. These results show that Mtb exert a strong intrinsic capacity to induce apoptosis in PMNs that is capable of overcoming the anti-apoptotic signaling in the PMN.

1. Introduction

The main infection route for *Mycobacterium tuberculosis* (Mtb) is through inhalation into the alveolar space, where the bacteria encounter alveolar macrophages and infiltrated polymorphonuclear granulocytes (PMNs) [1]. Being an intracellular bacterium without pronounced active invasion strategies, Mtb relies on phagocytosis by the host macrophages. To survive and be able to replicate or stay dormant within the macrophage, Mtb has to avoid intraphagosomal degradation and thus needs to modulate the response of the host cell by interfering with phagolysosome fusion [2], delaying apoptosis [3, 4] and render the cell insensitive to activation by IFN- γ [5].

PMNs that infiltrate the tissue infected with Mtb participate not only in removal of the bacteria [6], but also in the orchestration of inflammation through release of cytokines and chemokines [7]. PMNs have a short lifespan of 24-72 hours and apoptotic cells are usually cleared by macrophages, mediating an anti-inflammatory response [8]. Components of the Mtb cell wall or whole bacteria activate PMNs and induce apoptosis [7, 9, 10] but in contrast to spontaneously apoptotic PMNs, clearance of Mtb-induced apoptotic PMNs by macrophages is accompanied by a pro-inflammatory activation [11]. Hence, apoptotic PMNs are important cells in the initial innate immune reaction to Mtb infection and it is therefore vital to understand how Mtb induce apoptosis in inflammatory cells.

E. coli-induced production of reactive oxygen species (ROS) can oxidize the membranes of azurophilic granules resulting in leakage of cathepsins to the cytosol and propagation of the mitochondrial apoptosis pathway [12]. Virulent mycobacteria interfere with the fusion between granules and the phagosome [13, 14] but it is not known whether azurophilic granules are also involved in Mtb-induced PMN apoptosis.

Intracellular microbes *e.g. Salmonella typhimurium* induce apoptosis in phagocytic cells through modulation of phagocytic uptake and active invasion into the cell [15, 16]. However, when the same pathogen is ingested through immune receptors and resides in mature phago-lysosomes, no apoptosis occurs [16]. In fact, complement-mediated phagocytosis of *Salmonella typhimurium* activates PI-3 kinase and Akt in the phagocyte, thereby protecting the cell against apoptosis. In the present study we show how unopsonized Mtb induced apoptosis in PMNs. However, we found that also opsonized Mtb, despite being readily phagocytosed, induced apoptosis to a similar extent as unopsonized bacteria. This strong capacity to induce apoptosis was linked to expression of mature 19 kDa lipoproteins on Mtb, activation of MAP kinase, intracellular production of ROS, and subsequent mitochondrial damage in the PMNs.

2. Results

2.1 Opsonization of Mtb is required for efficient phagocytosis by PMNs. Several pathogens affect immune cells by surface interactions to avoid phagocytosis since phagocytosis of *e.g.* opsonized pathogens leads to killing of the ingested prey. We investigated whether phagocytosis *per se* was crucial for the effect of Mtb on cellular activation and apoptosis. Quenching of extracellular FITC-labeled bacteria with ethidium bromide followed by flow cytometric evaluation allowed us to monitor ingestion of Mtb by human PMNs. As illustrated in Fig. 1, 23% of the PMNs ingested opsonized Mtb, whereas <5% ingested unopsonized bacteria. Furthermore, unopsonized bacteria did not show any sustained interaction with the surface of the leukocytes (inset graph). Microscopic analysis of the same preparations verified the results obtained by flow cytometry.

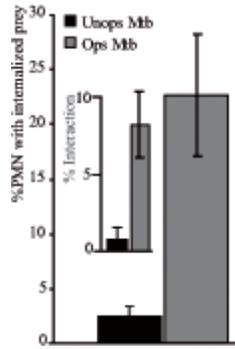


Figure 1 Only opsonized Mtb are phagocytosed.

Phagocytosis of Mtb was evaluated by the use of FITC-labeled bacteria. $23 \pm 6\%$ of PMNs phagocytosed opsonized Mtb (Ops Mtb), whereas only $3 \pm 1\%$ of PMNs ingested unopsonized Mtb (Unops Mtb). Data are presented as mean percentage \pm SD of the PMN population that internalized FITC-labeled Mtb. Inset graph represents surface interplay between PMN and FITC-Mtb i.e. Mtb associated with PMN. The data were derived from six separate experiments using PMNs from different donors.

2.2 Involvement of mitochondrial membrane damage. The intrinsic apoptotic cascade is mediated by release of apoptogenic intermembrane proteins such as cytochrome c from the mitochondria to the cytosol due to membrane rupture. This results in activation of caspases that execute the apoptosis. We found that mitochondrial membranes were significantly damaged in PMNs exposed to Mtb 7h post interaction, as indicated by changes in the mitochondrial membrane potential ($\Delta\Psi_m$) (Fig. 2). Both unopsonized and opsonized Mtb induced a decrease in the $\Delta\Psi_m$, indicating that activation of this intrinsic apoptotic pathway in human PMNs is not phagocytosis-dependent.

2.3 Mtb-induced apoptosis. The effect of Mtb on apoptosis in human PMNs was investigated to discern early molecular mechanisms involved in the host-pathogen interaction. Both unopsonized and opsonized Mtb triggered a significant increase in apoptosis in PMNs in a dose dependent fashion (Fig. 3A). Unopsonized and opsonized Mtb induced apoptosis to a level of 26 and 35% respectively compared to spontaneously apoptotic control cells (8%) (Fig. 3B), confirming previous findings [7, 11, 17]. Human PMNs infected with virulent Mtb displayed an

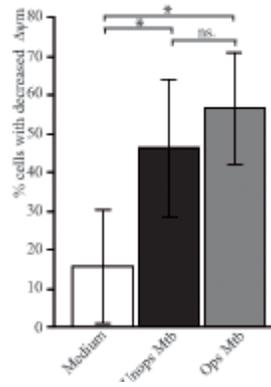


Figure 2 Mtb induce decreased mitochondrial membrane potential ($\Delta\Psi_m$). PMNs were exposed to unopsonized Mtb (Unops), opsonized Mtb (Ops), or medium alone (Medium) and $\Delta\Psi_m$ was evaluated 7h post interaction. Both unopsonized (Unops) and opsonized (Ops) Mtb induced significant decreases in $\Delta\Psi_m$ in PMNs as compared to control cells (Medium). The illustrated data represent mean percentage of the PMN population having decreased fluorescence (i.e., impaired $\Delta\Psi_m$) \pm SD from six separate experiments using PMNs from different donors. Significant differences between groups are indicated with * or ns (not significant).

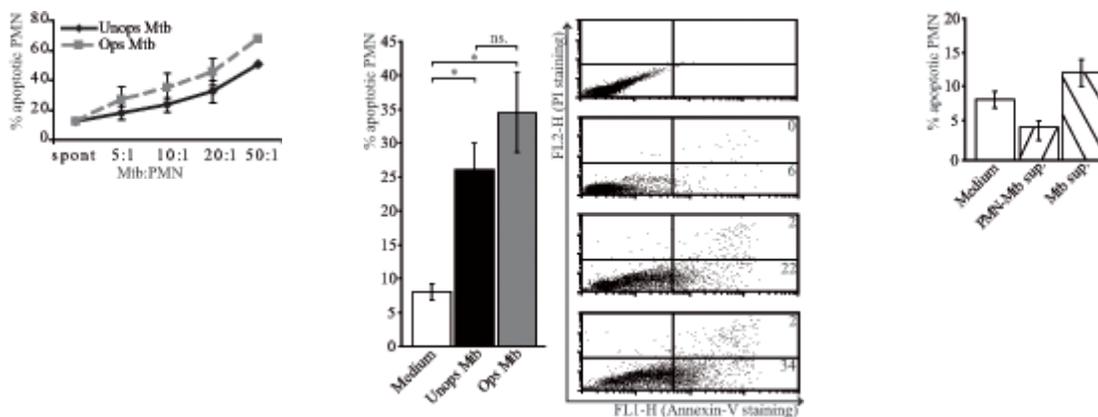


Figure 3 Interaction with Mtb induce apoptosis in PMNs without the involvement of soluble factors. PMNs were exposed to unopsonized (Unops Mtb) and opsonized (Ops Mtb) Mtb and dose-dependent apoptosis was detected (A). In addition, apoptosis was detected in the PMN population 7h post interaction with Mtb (B). To evaluate the plasma membrane integrity, all populations were counterstained with PI. Dot-plots show PI staining (FL2-H) and annexin-V staining (FL1-H) of (top to bottom); unstained cells or PMNs stimulated with; medium, unopsonized Mtb or opsonized Mtb (B). The percentage of the population staining positive is indicated in respective quadrants of interest. To determine the involvement of soluble factors to PMN apoptosis, freshly isolated PMNs were incubated in culture supernatants derived from Mtb alone (Mtb sup.), or from Mtb-induced apoptotic PMNs (PMN-Mtb sup.) (C). Data are presented as mean percentage \pm SD of the PMNs population staining positive for phosphatidyl serine exposure. The data were derived from six separate experiments (three for supernatant stimulation and dose response) using PMNs from different donors. In figure 3A differences between untreated (Medium) and stimulated samples are depicted as * or ns (not significant).

apoptotic response with externalized phosphatidyl serine and sustained plasma membrane integrity (Fig 3B). Since phagocytic uptake of Mtb was very limited and did not correlate with the number of apoptotic cells, we investigated whether soluble factors were involved in the initiation of apoptosis. Freshly isolated PMNs were cultured in culture supernatants derived from Mtb or from Mtb-induced apoptotic PMNs. Neither of these treatments led to any significant increase in apoptosis (Fig. 3C) indicating that a Mtb-cell or cell-cell interaction was necessary for the induction of apoptosis.

2.4 Mtb lipoproteins are important for induction of apoptosis. Since apoptosis in PMNs requires Mtb-cell contact but not phagocytosis of Mtb, we explored if certain surface-expressed bacterial determinants were involved in the apoptosis induced by this pathogen. Since purified 19 kDa lipoprotein from Mtb can activate and prime human PMNs [18], we investigated whether a *lspA*^{-/-} Mtb mutant lacking the mature cell wall component 19 kDa lipoprotein displayed altered capacity to induce PMN apoptosis. Indeed, we found that the mutant *lspA*^{-/-} was less potent in inducing apoptosis in PMNs compared to the parent strain (wt) (Fig. 4A), despite the fact that the mutant bacterium interacted with the leukocyte and induced production of ROS to the same extent (data not shown). Additionally, opsonization of the mutant *lspA*^{-/-} strain did not affect its capacity to induce apoptosis (data not shown). To restore the lack of mature lipoproteins on the mutant *lspA*^{-/-} strain, the bacteria were pre-incubated with the triacylated Pam₃CysSK₄ mimicking the 19 kD lipoprotein. Following this restoration of the bacterial cell wall component the mutant *lspA*^{-/-} strain regained apoptosis-inducing capacity to levels similar to wt bacteria. Pam₃CysSK₄ alone did not affect the apoptosis in PMNs and presence of Pam₃CysSK₄ did not modify the apoptotic response of PMNs to wt Mtb (Fig 4A). Despite that Pam₃CysSK₄ has been described as a potent TLR2 ligand [19], indicating involvement of TLR2

in Mtb-induced apoptosis, blocking with anti TLR2 antibodies did not affect the induction of apoptosis (Fig 4B).

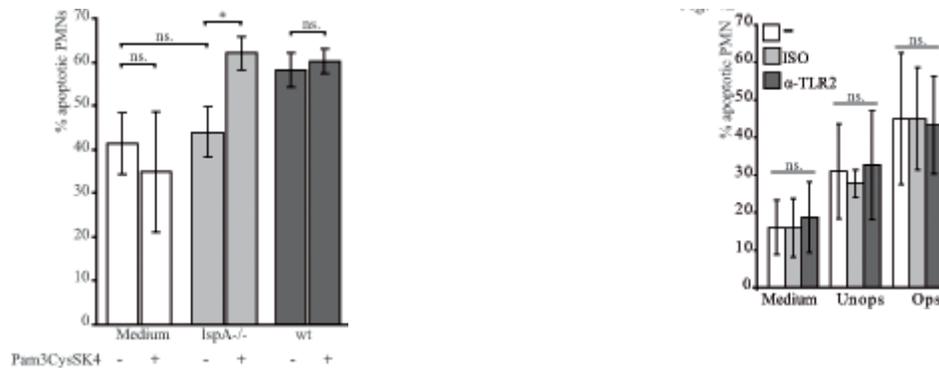


Figure 4 Mature lipoproteins are crucial for Mtb to induce apoptosis in PMNs but without involvement of TLR2. PMNs were exposed to *lspA*^{-/-} mutant Mtb (*lspA*^{-/-}), a strain deficient in producing mature lipoproteins e.g. the 19kDa lipoprotein, or parental strain (wt) (A). Complementation of the *lspA*^{-/-} with the triacylated Pam3CysSK₄ restored the apoptosis inducing properties, whereas Pam3CysSK₄ did not affect the apoptosis in neither control cells nor in cells stimulated with wt bacteria. Further, PMNs were stimulated with medium (Medium), unopsonized Mtb (Unops), or opsonized Mtb (Ops) in the presence of TLR2 blocking antibodies (α-TLR2) or iso type control antibody (ISO) (B) and apoptosis was detected following 7h incubation. Data are expressed as mean percentage ± SD of the PMN population staining positive for phosphatidyl serine exposure. The illustrated data are derived from five separate experiments (A), and four separate experiments (B) respectively, using PMNs from different donors. Significant differences between groups are indicated with * or ns. (not significant).

2.5 Phosphorylation of pro-survival Akt and pro-apoptotic p38 MAPK.

Opsonization-dependent phagocytosis of *Salmonella typhimurium* results in phosphorylation of the apoptosis-protective protein Akt giving the activated protein the capacity to reduce apoptosis [16]. We therefore addressed the question how pro- and anti-apoptotic signals were induced in PMNs by Mtb and found that Akt was activated by opsonized Mtb (Fig. 5A). It is furthermore known that phosphorylated p38 MAPK plays an important role in the regulation of apoptosis, by inducing pro-apoptotic events [20]. We found that p38 MAPK was activated to similar extent in PMNs exposed to opsonized and unopsonized Mtb (Fig. 5A), indicating that Mtb-induced activation of p38 MAPK in PMNs is contact dependent but not phagocytosis dependent.

Inhibition of Akt activation did however not enhance Mtb-induced apoptosis (Fig. 5B), showing that activation of Akt is not sufficient to counteract apoptosis mediated by opsonized Mtb. In contrast, inhibition of p38 MAPK impaired the pro-apoptotic effect of Mtb on PMNs (Fig. 5B). By comparison, inhibition of ERK and JNK, which are also members of the MAP kinase family, did not affect the induction of apoptosis, suggesting that these pathways are not involved in regulation of Mtb-induced apoptosis in human PMNs (data not shown).

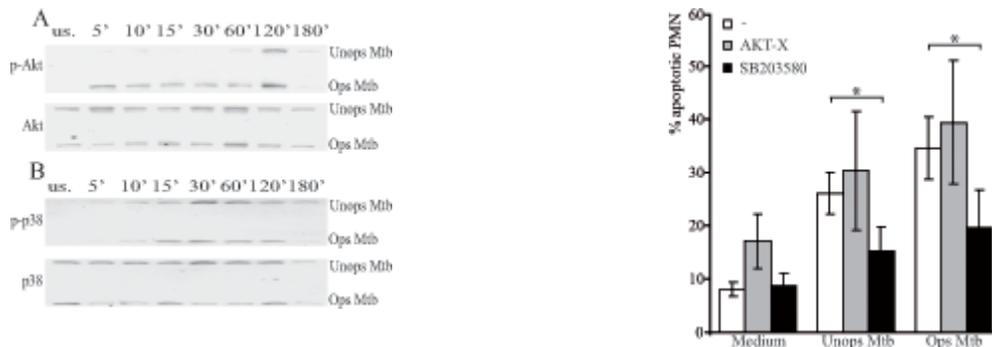


Figure 5 Both Akt (p-Akt) and p38 MAPK (p-p38) are upregulated in Mtb-exposed PMNs, but only p38 affect Mtb-induced apoptosis. PMNs were exposed to unopsonized (Unops Mtb), or opsonized (Ops Mtb) Mtb whereafter Akt (p-Akt) (A) and p38 MAPK (p-p38) (B) were detected by Western blot technique. The first lane in each blot shows activation in unstimulated PMNs (us.), followed by PMNs exposed to Mtb for 5 to 180 min. All blots were subsequently stripped and re-probed for total Akt (Akt) or p38 MAPK (p38) to ensure equal loading of the samples. Further, to evaluate the involvement of these proteins, PMNs were pre-incubated with Akt-X (Akt inhibitor) or SB203580 (p38 inhibitor) prior to induction of apoptosis by unopsonized (Unops Mtb) or opsonized (Ops Mtb) Mtb (C). The blots have been digitally contrast enhanced and are representative of six separate experiments using PMNs from different donors. In Fig. 5C, the illustrated data represent apoptosis of the PMN population \pm SD given as means of six separate experiments using PMNs from different donors. Significant differences between groups are indicated with *.

2.5 Involvement of ROS production. It has been shown that ROS produced during cell activation is a mediator of apoptosis in inflammatory cells [12, 21]. Using luminol-amplified chemiluminescence, we measured formation of ROS in PMNs and found that both opsonized and unopsonized Mtb induced similar amount of ROS (Fig. 6A). However, despite similar total amount of ROS, the localization of ROS production differed. In PMNs exposed to opsonized

Mtb, ROS was initially generated intracellularly while in PMNs exposed to unopsonized Mtb, the majority of the ROS was extracellular (data not shown). As shown in Fig. 6B, the respiratory burst induced by Mtb was weaker (<15%) than that elicited by an *E. coli* strain (FimH+, expressing type 1 fimbriae), previously reported to cause strictly ROS-dependent apoptosis [12]. On the other hand, the low level of ROS production in response to Mtb was sustained for 2–3 h. Furthermore, despite the limited ROS formation, apoptosis was dependent on activity of NADPH oxidase, since DPI (an inhibitor of NADPH oxidase) treatment of PMNs abrogated the ROS production (Fig 6A) and both unopsonized and opsonized Mtb failed to induce apoptosis in DPI treated PMNs (Fig. 6C). In addition, Western blot analysis of p38 MAPK phosphorylation in DPI treated cells revealed that p38 activation was not affected by inhibition of NADPH oxidase (data not shown).

2.6 Lysosomal membrane permeabilization is not involved in the induction of apoptosis. One of the initial targets for intracellular ROS is the membrane of lysosomal granules. Oxidative damage of these membrane leads to permeabilization of the granules and leakage of lysosomal cathepsins to the cytosol [12, 22, 23], events closely linked to amplification of apoptotic signals. Therefore, we measured the integrity of these granules in PMNs subjected to Mtb-induced apoptosis, by monitoring cells with intact acridine orange-accumulating lysosomes (Fig. 7). We found no significant lysosomal damage in Mtb-induced apoptotic PMNs.

3. Discussion

PMNs respond to mycobacteria by exhibiting characteristic bactericidal activity involving phagocytosis [24, 25], activation of NADPH oxidase with subsequent ROS production [26, 27], exocytosis of specific granules [13], and killing of mycobacteria through oxidative or non-

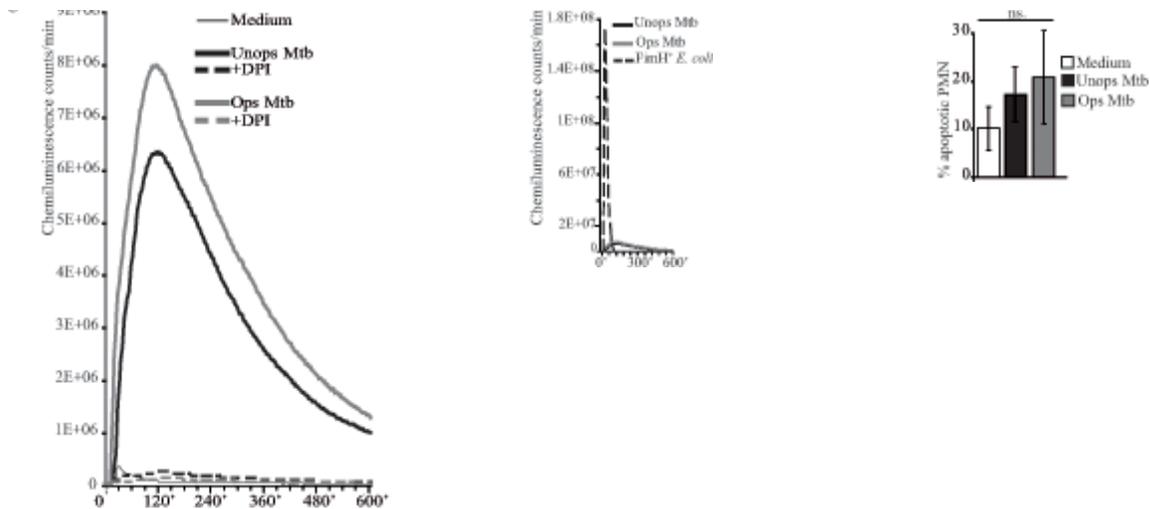


Figure 6 *Mtb* triggers production of ROS in PMNs. Using luminol-enhanced chemiluminescence, ROS was continuously measured in PMNs for 300 min following interaction with unopsonized (Unops), opsonized (Ops) (A) or *E. coli* FimH+ (B). DPI abrogated the *Mtb*-induced ROS production (A) and apoptosis (C) in PMNs. Chemiluminescence data represent means of six separate experiments. The apoptosis in the PMN population (C) is given as mean \pm SD for six separate experiments using PMNs from different donors. Lack of significant difference between DPI treated, *Mtb* stimulated cells and medium control is indicated with ns. (not significant).

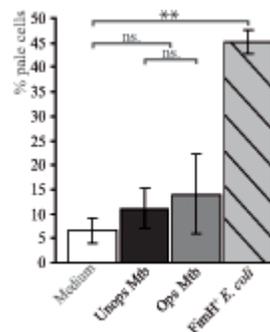


Figure 7 *Mtb*-induced apoptosis in PMNs is not due to permeabilization of lysosomal membranes. PMNs were exposed to unopsonized (Unops *Mtb*) or opsonized (Ops *Mtb*) *Mtb*, the positive control (FimH+ *E. coli*), or medium alone (Medium) whereafter the lysosomal membrane integrity was measured 6 h after exposure to the bacteria (A). The illustrated data represent mean percentage of the PMN population consisting of pale cells (i.e. loss of lysosomal membrane integrity) \pm SD from six separate experiments using PMNs from different donors. Significant differences between groups are indicated with * or ns (not significant).

oxidative mechanisms [6, 25, 28, 29]. Although PMN are considered the main cell in the early phase of inflammation, it is not until recently that this cell population has been described to play

an important role during chronic infection and tuberculosis [30]. Immune receptor-mediated phagocytosis of virulent bacteria has been demonstrated to induce anti-apoptotic proteins (Akt and MAPK/ERK) and reduce apoptosis [16], but despite this, PMNs enter apoptosis following interaction with opsonized Mtb. However, unopsonized Mtb induced apoptosis in PMNs to the same extent without being phagocytosed. This indicates that Mtb has a strong intrinsic potential to induce apoptosis in PMNs and is able to overcome the anti-apoptotic Akt-dependent mechanisms following immuno-mediated phagocytosis.

The fact that Mtb-induced apoptosis of PMNs triggers chemokine expression [7] and heat shock protein release [11], and the finding that the number of apoptotic cells (26%) exceeded the number of phagocytosing cells (<5%), suggest that soluble factors are involved in the induction of apoptosis. However, we were unable to detect any direct effects of soluble factors from either Mtb or Mtb-induced apoptotic PMNs. The lack of apoptogenic factors in supernatants from Mtb-induced apoptotic PMNs implies that the apoptosis is the result of a direct interaction between the mycobacteria and the PMNs, possibly through specific interactions with TLRs or other recognition sites [31]. Recently, Kelly *et al.* (2007) showed that bystander macrophages adjacent to infected macrophages entered apoptosis in a contact dependent fashion without involvement of soluble factors [32]. In the present study, similar activation of bystander cells may be executed by low numbers of activated PMNs.

The PI-3 kinase/Akt pathway is activated by a variety of stimuli [33, 34], one of which is immune receptor-mediated phagocytosis of opsonized bacteria [16, 35]. The anti-apoptotic mechanisms utilized by Akt involves an increase in the Mcl-1–Bax interaction, which augments the sequestration of pro-apoptotic Bcl-2 proteins preventing them from initiating the mitochondrial pathway [36]. Mtb-induced apoptosis in PMNs was however not impaired despite early up-regulation of the survival signal Akt when challenged with opsonized Mtb, suggesting that Mtb

has a unique and strong intrinsic capacity to trigger apoptosis, despite activation of anti-apoptotic proteins. This conclusion is supported by our observation that inhibition of Akt did not enhance PMN apoptosis. Phosphorylated p38 MAPK is involved in an abundance of cellular signalling cascades involving activation and regulation of ROS production by NADPH oxidase in PMNs [27, 37, 38]. We found that p38 MAPK was a key mediator in Mtb-induced apoptosis. This finding may be explained by upstream modulation of NADPH oxidase activity since inhibition of p38 phosphorylation results in failed ROS production [27] whereas inhibition of NADPH oxidase does not affect p38 activity. The involvement of p38 is consistent with findings reported by Aleman *et al.* (2004), who showed that p38 MAPK was activated in circulating PMNs from patients with tuberculosis, and that these PMNs showed accelerated apoptosis compared to uninfected control cells [31]. However, it is unlikely that circulating PMNs have come in contact with Mtb since the bacteria mainly reside in the lung tissue. Aleman *et al.* (2004) also found that Mtb-induced apoptosis could be prevented by blocking anti-TLR2 antibodies in PMNs from tuberculosis patients, but not in cells from healthy controls. This supports our finding that pretreatment of PMNs with blocking TLR antibodies did not affect the induction of apoptosis by Mtb.

Apoptosis elicited by opsonized Mtb is dependent on ROS production by the NADPH oxidase [11, 17]. The present experiments show that apoptosis mediated by unopsonized Mtb also requires intracellular ROS, as indicated by the findings that Mtb-induced apoptosis was abrogated by DPI, and that the extent and kinetics of the ROS production were similar for opsonized and unopsonized bacteria. Nevertheless, the formation of ROS was quite limited as compared to levels induced by other strong apoptotic stimuli, such as FimH+ *E. coli* [12]. The fact that the limited ROS response was prolonged and did not reach the basal level until after 10 h, indicates that Mtb-induced ROS production have signaling properties rather than serving as an early

executor of apoptosis. However, the *lspA*^{-/-} strain, lacking mature lipoproteins, induced similar low amounts of ROS but failed to induce apoptosis, indicating that ROS is not the sole trigger of apoptosis, and that additional Mtb-specific signals are required.

Intracellular ROS such as H₂O₂ oxidizes lipids in granule membranes [22], resulting in leakage of cathepsins from the vacuole and activation of the apoptotic cascade via the mitochondrial pathway. Our results showed that, even though PMNs that phagocytose Mtb are left with large numbers of unfused primary granules [14], the moderate ROS production induced no lysosomal damage. However, the mitochondria were central in the apoptosis induction and $\Delta\Psi_m$ was significantly reduced, supporting our previous observation that expression of antagonizing Bcl-2 proteins is altered early during Mtb-induced apoptosis [17]. Low levels of oxidative stress induce apoptosis in several cell types, and ROS or lipid peroxidation products may do so through the redox sensitive mitochondrial permeabilization pore [39, 40].

Our finding that the *lspA*^{-/-} mutant did not provoke apoptosis supports the view that mature lipoproteins are necessary for induction of apoptosis also in PMNs, as has previously been described in macrophages using recombinant 19kD lipoprotein [41]. This is further supported by complementation of the *lspA*^{-/-} mutant with triacylated Pam₃CysSK₄, mimicking ligands such as 19kD, which restored the apoptosis-inducing capacity. Additionally, the restored apoptosis induction was not a result of increased ROS production since Pam₃CysSK₄ did not affect NADPH oxidase activity.

An efficacious Mtb-specific, CD4 T-cell-mediated immune response is orchestrated primarily in the lymph nodes [42], and Abadie *et al.* (2005) demonstrated that PMNs can shuttle BCG bacteria from the site of infection to draining lymph nodes [43]. Therefore, it would be important to follow the role of apoptotic PMNs during granuloma formation, especially considering our previous observations that Mtb-induced apoptotic PMNs initiates a pro-inflammatory response in

macrophages [11], and induces DC maturation (unpublished observations). The recent observation that Mtb can trigger local tissue macrophages to further attract peripheral cell infiltration [44] supports the view that tuberculosis is a complex infection affecting not only the local immune response, but cell traffic as well.

3. Conclusions

Mtb exert a strong intrinsic capacity to induce apoptosis in human PMNs capable of overcoming anti-apoptotic signals initiated in the cell. Furthermore, the Mtb-induced apoptosis does not require phagocytosis or sustained interaction with the cell despite lack of soluble factors present. This may be the result of transient interaction between the PMN and Mtb sufficient of inducing apoptosis or that Mtb-induced apoptotic cells in turn affect adjacent cells in the population. In a larger perspective, Mtb-induced apoptotic cells, in contrast to senescent PMNs, activate macrophages [11], and PMNs shuttle BCG bacteria to draining lymph nodes [43]. Whether the induction of apoptosis in PMNs by Mtb is a virulence or defense mechanism remains to be further investigated. Although apoptotic cells are incapable of ingesting or killing microbes, they may still serve a function as innate immune modulators. Taken together, Mtb-induced apoptosis in PMNs is not necessarily detrimental to the host, but serves as an important link between innate and adaptive immune responses to Mtb during the early phase of infection.

4. Materials and methods

4.1 Materials. The chemicals and their sources were as follows: acridine orange (AO), diphenylene iodonium (DPI) and tetramethylrhodamine ethyl ester perchlorate (TMRE) were purchased from Sigma-Aldrich. (St Louis, MO, USA); Pam₃Cys-SK₄ was purchased from EMC

microcollections (Tübingen, Germany); anti-phospho p38 MAPK (Thr180/Tyr 182), anti-p38 MAPK, anti-phospho Akt (Ser473), and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA); HRP-labeled goat anti-mouse and anti-rabbit antibodies were from DakoCytomation (Glostrup, Denmark); JNK inhibitor II, SB203580, Akt inhibitor X, and PD 98059 were from Calbiochem (San Diego, CA); TLR2 antibody and isotype control were purchased from eBiosciences (San Diego, CA); an annexin-V apoptosis detection kit was obtained from R&D Systems (Abingdon, UK); FBS (fetal bovine serum), L-glutamine, and RPMI 1640 cell culture media were from Gibco (Grand Island, NY); Polymorphprep[®], Lymphoprep[®], Tween-20 and ECL reagents were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). PBS (137 mM NaCl, 2.7 mM KCl, 6.7 mM Na₂HPO₄, and 1.5 mM KH₂PO₄; pH 7.3) was prepared in-house. Heparinized peripheral blood was obtained from the blood bank at Linköping University Hospital, Linköping, Sweden.

4.2 Cell isolation and culture. Human PMNs were isolated from heparinized peripheral blood from healthy donors by density-gradient separation on Polymorphprep[®] as described elsewhere [27]. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat-inactivated FBS (RPMI) was used in PMN incubations and experiments.

4.3 Bacteria. The γ -inactivated *Mycobacterium tuberculosis* strain H37Rv was kindly provided by Colorado State University (Fort Collins, CO), and H37Rv *lspA*^{-/-} and wildtype (wt) were gifts from Dr. Joel Ernst, New York University School of Medicine, New York, NY. The mutant *lspA*^{-/-} and wt were cultured statically in Middlebrook 7H9 supplemented with ADC, 0.05% tween-80, and 0.5% glycerol for 14 days and were subsequently heat-inactivated at 80°C for 60 min. Single-cell suspensions of all bacteria was prepared using a Dounce homogenizer, sedimentation and multiple passages through a 27 gauge syringe and stored at -70°C. Before use,

aliquots were passed multiple times through a 27 gauge syringe and some were opsonized in 50% NHS for 30 min.

4.4 Interaction and phagocytosis. Interaction with and/or phagocytosis of Mtb by PMNs was detected using FITC-labeled bacteria and was subsequently quantified microscopically by the trypan blue exclusion method or flow cytometry, as described elsewhere [45]. This is an extensively used and reliable technique that distinguishes between attached and ingested bacteria [46].

4.5 Mitochondrial membrane damage. Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed as accumulation of tetramethylrhodamine ethyl ester (TMRE) as previously described [12]. Briefly, PMNs were incubated in 1 μ M TMRE for 15 min at 37°C to allow accumulation of the TMRE in the mitochondrial matrix. Decreased $\Delta\Psi_m$ was indicated by a reduction in the TMRE red fluorescence, as measured by flow cytometric analysis.

4.6 Apoptosis. Freshly isolated PMNs were allowed to interact with Mtb for 45 min at 37°C at a ratio of 10 Mtb per PMN unless otherwise stated in the text followed by three brief washes in cold PBS to remove non-ingested bacteria, and left at 37°C to allow apoptosis. The amount of apoptosis in the PMN population was detected by staining the cells with FITC-conjugated annexin-V and then counterstaining with propidium iodide to detect late apoptosis and/or necrosis according to manufacturer's protocol. No samples were detected to have >5% propidium iodide staining (data not shown) and were thus identified as apoptotic and not necrotic. Analysis was performed by flow cytometry with Cell-Quest software (FACSCalibur, BD Biosciences, San Jose, CA). In some experiments the PMNs were pre-treated with inhibitors to block certain signaling pathways and effector systems: PD98059 (25 μ M) against ERK (p42/44 MAPK), Akt-X (10 μ M) against Akt, SB203580 (10 μ M) against p38 MAPK, or JNK inhibitor II

(5 μ M), or DPI (5 μ M) for 30 min at 37°C. To restore the exposure of acylated lipoproteins the *IspA*^{-/-} or wt bacteria were pre-incubated with triacylated Pam₃CysSK₄ (1 μ g/ml for 20 min RT) before they were resuspended in medium and presented to the PMNs. Also, in some experiments TLR2 was blocked by incubation with 30 μ g/ml of specific antibodies or isotype control for 30 min as previously described [31]. The ability of the antibody to block TLR2 ligation was established by challenging macrophages with PamCys and evaluation of NF κ B translocation to the nucleus.

4.7 Expression and activation of Akt and p38 MAPK. PMNs (2×10^6) were exposed to Mtb as described earlier, and incubated at 37°C, whereafter cellular processes were stopped by addition of ice cold PBS. The cells were then washed once and boiled in Laemmli sample buffer for 10 min. Activation of Akt and p38 MAPK was detected by Western blotting using anti-phospho Akt (Ser473) and anti-phospho p38 MAPK (Thr180/Tyr 182) specific antibodies. Antibodies against total Akt and p38 MAPK were used to ensure equal loading of the samples.

4.8 Chemiluminescence detection of ROS. One of the hallmark responses in PMN activation is the production of ROS. We used luminol-amplified chemiluminescence (20 μ g/ml luminol and 4 U/ml HRP) to measure the ROS generated by the PMNs after exposure to Mtb. To distinguish between intra- and extra-cellular ROS, catalase and superoxide dismutase was used to scavenge extracellular ROS. Also in some experiments, PMN were pre-treated with DPI (5 μ M) for 30 min at 37°C.

4.9 Lysosomal membrane stability. Lysosomal stability was assessed by the acridine orange (AO) uptake method as previously described [12, 47]. Briefly, at the end of all experiments, the cells were allowed to accumulate AO by incubation in 5 μ g/ml AO in RPMI for 15 min at 37°C. Pale cells (cells that displayed a reduced number of intact AO-accumulating lysosomes) exhibited decreased red fluorescence when analyzed with flow cytometry using

CellQuest software.

4.10 Analysis of data. The data presented in text and figures are expressed as means \pm SD. Differences between groups were calculated by student's t-test and were considered significant at $p < 0.01$ and denoted as * or ns. (not significant).

5. Acknowledgments

Mycobacterium tuberculosis H37Rv was kindly provided by Colorado State University according to NIH: NO1-AI-40091. The authors thank Patricia Ödman for language revisions. This work was supported by the Swedish Research Council (5968), Sida/SAREC, the Swedish Heart Lung Foundation, the County Council of Östergötland and the Söderberg foundation.

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