ESX-1-induced apoptosis is involved in cell-to-cell spread of Mycobacterium tuberculosis.

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ESX-1-induced apoptosis is involved in cell-to-cell spread of *Mycobacterium tuberculosis*.

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ABSTRACT

Apoptosis modulation is a procedure amply utilized by intracellular pathogens to favour the outcome of the infection. Nevertheless, the role of apoptosis during infection with *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, is subject of an intense debate and still remains unclear. In this work, we describe that apoptosis induction in host cells is clearly restricted to virulent *M. tuberculosis* strains, and is associated with the capacity of the mycobacteria to secrete the 6-kD early secreted antigenic target ESAT-6 both under *in vitro* and *in vivo* conditions. Remarkably, only apoptosis-inducing strains are able to propagate infection into new cells, suggesting that apoptosis is used by *M. tuberculosis* as a colonization mechanism. Finally, we demonstrate that *in vitro* modulation of apoptosis affects mycobacterial cell-to-cell spread capacity, establishing an unambiguous relationship between apoptosis and propagation of *M. tuberculosis.* Our data further indicate that BCG and MTBVAC vaccines are inefficient in inducing apoptosis and colonizing new cells, correlating with the strong attenuation profile of these strains previously observed *in vitro* and *in vivo*.

INTRODUCTION

Tuberculosis represents a menace to global human health, causing more than one million deaths per year, and being one of the leading infectious diseases affecting developing countries (WHO, 2012). *Mycobacterium tuberculosis*, the causative agent of the disease, is primarily an intracellular pathogen that has successfully developed strategies to colonize alveolar host macrophages and overcome their bactericidal defence mechanisms. This permits bacterial replication and propagation in the host during the early stages of the infection, in the absence of an organized protective response capable to control infection (Cooper, 2009).

ESAT-6, which is secreted via the ESX-1 secretion system, is an immunodominant antigen involved in virulence. ESAT-6 has been implicated in different host-pathogen interaction processes leading to downmodulation of macrophage activity (Novikov et al, 2011; Pathak et al, 2007), autophagy inhibition (Romagnoli et al, 2012) or phagosome membrane disruption, which allows *M. tuberculosis* to translocate to cytosol (Houben et al). *Esat-6* is encoded in the region of difference 1 (RD1), which is deleted from BCG. Although BCG's genome contains different RDs that codify for genes potentially involved in virulence (Gordon et al, 1999), RD1 deletion has been described as the main cause for the attenuation profile of BCG (Pym et al, 2002).

Apoptosis is a physiological type of cell death characterized by the preservation of the plasma membrane integrity. Apoptotic bodies express "eat-me" signals recognized by macrophages to become phagocytosed (Martin et al, 1995). Thus, release of intracellular content to the extracellular medium, as well as associated inflammatory reactions, is prevented. Modulation of host cell death as a mechanism to overtake host defences is a strategy amply exploited by intracellular bacteria. In the case of Chlamydia, an obligate intracellular type of bacteria, infected host cells are profoundly resistant to apoptosis (Fan et al, 1998). Conversely, other facultative intracellular pathogens such as *Shigella* (Zychlinsky et al, 1992) or *Salmonella* (Monack et al, 1996) cause host cell apoptosis. In the case of *M. tuberculosis*, the role of apoptosis for the infection outcome is subject to an intense debate. Several works maintain that capacity to induce apoptosis is characteristic of attenuated mycobacterial strains (Chen et al, 2006; Sly et al, 2003). Thus, apoptotic macrophages would provide mycobacterial antigens to

be processed and presented by dendritic cells (Schaible et al, 2003). On the contrary, other authors describe that apoptosis is induced exclusively by virulent *M. tuberculosis* strains, both *in vitro* and *in vivo* (Aporta et al, 2012; Seimon et al, 2010), in a process that involves ESAT-6 (Derrick & Morris, 2007; Grover & Izzo, 2012). The finding that inhibition of *Mycobacterium marinum*-induced apoptosis impairs the spread of infection (Davis & Ramakrishnan, 2009) further suggests that induction of apoptosis seems to be a potent virulence mechanism of pathogenic mycobacteria. Recently, we showed that the current BCG vaccine and the attenuated *M. tuberculosis* SO2 prototype vaccine candidate (Martin et al, 2006) were unable to induce apoptosis in infected macrophages, both under *in vitro* and *in vivo* conditions (Aporta et al, 2012). These findings suggest that induction of apoptosis is a key mechanism used by the pathogen that is apparently lost by attenuated strains unable to secrete ESAT-6.

MTBVAC is a recombinant live vaccine candidate, derived from the *M. tuberculosis* clinical isolate MT103, attenuated by the deletion of the virulence genes *phoP* and *fadD26*, and is the first such candidate to be tested in human clinical trials (Arbues *et al.* Submitted for publication). PhoP is part of the two-component system PhoPR, which regulates the transcription of approximately 2% of *M. tuberculosis* genome (Gonzalo-Asensio et al, 2008; Walters et al, 2006), with several of the PhoP-regulated genes involved in virulence mechanisms, including ESAT-6 secretion (Frigui et al, 2008). *FadD26* is essential for the synthesis and the transport to the bacterial surface of phthiocerol dimycocerosates (PDIM/DIM), a lipid complex involved in virulence (Camacho et al, 1999; Cox et al, 1999).

In this work we have employed a panel of different virulent *M. tuberculosis* strains, including various clinical isolates, to analyse their ability to induce apoptosis in comparison with the attenuated mycobacterial vaccines BCG and MTBVAC. In addition, we explore whether apoptosis induction leads to mycobacterial cell-to-cell spread.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Mycobacteria used in this study were grown at 37°C in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.05% Tween 80 and 10% Middlebrook albumin dextrose catalase enrichment (ADC; BD Biosciences) and, when required, medium was supplemented with 20 µg/ml of kanamycin or hygromycin. GFP-expressing strains were generated by transformation of plasmid pMV361H *gfp* (Green Fluorescent Protein). Representative Beijing *M. tuberculosis* clinical isolates selected in European Project TB-VIR were used (Wang et al, 2010).

CELL CULTURE AND INFECTIONS

MH-S cells (HPA) were cultured at 37°C and 5% CO_2 in DMEM medium supplemented with 10% inactivated foetal bovine serum (Biological industries) and 2 mM glutamine (Biological industries). Cells were seeded in 24-plate wells and allowed to attach to the plastic overnight. After clumps removal by low-speed centrifugation of a log-phase culture, bacterial concentration was determined by optical density. Bacterial suspension for indicated MOIs was prepared in DMEM complete medium and put in contact with cells for 4 h. Afterwards, cells were washed three times with PBS to remove extracellular bacteria and fresh DMEM complete medium was added, in the presence of SB202190 inhibitor (Merck Millipore) or staurosporine (0.025, 0.05, 0.1, 0.2, 0.5 µM) (Sigma) when indicated.

APOPTOSIS ANALYSIS *IN VITRO*

Phosphatidylserine (PS) exposure and plasma membrane integrity were evaluated by AnnexinV-APC (AnnV) and 7-actinomycinD (7-AAD) (BD Biosciences) staining according to manufacturer instructions, and analyzed by flow cytometry. Briefly, cells were washed and incubated with AnnV and 7AAD in Annexin-binding buffer (ABB) for 15 min in dark at room temperature. Afterwards, cells were washed with ABB and fixed with 4% paraformaldehyde (PFA) containing $CaCl_2$. Nuclear morphology was analyzed by fluorescence microscopy with Hoechst 33342 (Invitrogen), according to manufacturer instructions.

IN VIVO STUDIES IN MICE

The protocols for animal handling were previously approved by University of Zaragoza Animal Ethics Committee (protocol number PI43/10). Eight weeks old female C57BL/6 mice were intranasally challenged with approximately 1000 CFUs of the indicated strains suspended in 40 µl of PBS. Four weeks post-infection, animals were humanely sacrificed and lungs and/or spleen were harvested.

To analyze bacterial replication, lungs or spleen were homogenized using GentleMacs homogeneizer (Miltenyi Biotec) and CFU counted by plating serial dilutions on solid Middlebrook 7H11 medium supplemented (BD Biosciences) with 10% Middlebrook ADC enrichment.

Histological and immunohistochemical protocols were performed according to a previous work (Aporta et al, 2012). Lungs were harvested and fixed in 4% Neutral Buffered Formalin, placed into Histology cassettes and processed in the Xpress X50 rapid tissue processor (Sakura, Japan) until paraffin embedding. Paraffin blocks were made and cut at 3 um. Sections were stained with Hematoxylin-Eosin and Ziehl-Nielsen stain methods for histological assessment. For immunohistochemistry, sections were deparaffinized in xylene and hydrated in a gradient alcohol series from 100% to 70% and running water for 5 minutes. Heat mediated antigen retrieval was performed by means of PT-Link (Dako, Denmark) by heating the slides at 92°C in low or high pH buffer (Target Retrieval Solution, High pH or Low pH, Dako, Denmark) depending on the antibody, for 20 min and then washed in wash buffer (Dako, Denmark). Endogenous peroxidase was quenched (Peroxidase-Blocking Reagent, EnVision™, Dako, Denmark) followed by incubation with Caspase-3 active (R&D systems) and F4/80 (Abcam) primary antibodies. For visualization, Dako EnVision System HRP was used depending on the antibody with a suitable secondary antibody (HRP labelled goat anti rabbit or rabbit anti rat) following suppliers procedure. The colour reaction was developed by DAB+ chromogen in substrate buffer (Dako, Denmark), resulting in a brown reaction product. Sections were counterstained with Mayer's hematoxylin, dehydrated in a gradient series of alcohol, cleared in xylene and mounted. In negative controls, the primary antibody was omitted.

For histological analysis, the whole lung of each animal was studied with a Leica DM5000B microscope and representative pictures of each slide taken with a Leica DFC 420C camera at indicated magnification. Histological findings and positive labelled cells and location compared to negative controls were assessed and recorded.

WESTERN BLOT ANALYSIS

Supernatants from the indicated strains were obtained by filtration and TCA precipitation of 10 ml log-phase cultures. Protein concentration was determined by Bradford method (BioRad) and 10 µg total protein were loaded in a 15% polyacrylamide gel, separated by SDS-PAGE and transferred to PVDF membrane (GE Healthcare). Membranes were incubated with anti-ESAT6 (Abcam) or anti-Ag85A (Abcam) primary antibodies. After corresponding secondary antibodies incubation, membranes were revealed using ECL plus Western Blotting system (GE Healthcare).

STATISTICAL ANALYSIS

Statistical analysiswere performed with the GraphPrism software, using indicated tests. Differences were considered significant at $p<0.05$.

RESULTS

APOPTOSIS INDUCTION IS RESTRICTED TO ESAT-6-SECRETING STRAINS

In a previous study, we showed that the clinical *M. tuberculosis* isolate MT103 triggered apoptosis in infected macrophages (Aporta et al, 2012). To find out whether this is a general feature of virulent *M. tuberculosis* strains, we analyzed the pro-apoptotic capacity of different strains, including the reference strain H37Rv, MT103 and eight clinical isolates belonging to the Beijing family (Wang et al, 2010). In this work, we used as representative host cell model the MH-S cell line, comprising immortalized murine alveolar macrophages (Mbawuike & Herscowitz, 1989), thus representing an attractive model to study the interaction of this pathogen with the host cell. This cell line has been characterized during mycobacterial infection and has been evaluated in comparison with primary alveolar macrophages, showing a comparable expression of surface markers and a similar capacity to interact with mycobacteria (Melo & Stokes, 2000). As shown in Figure 1A, the different Beijing family clinical isolates (strains W4, N4, NHN5, GC1237, HM764, HM903, CAM22 and 990172) induced cell death on MH-S cells to a similar extent as MT103 or H37Rv. The phenotype observed corresponded clearly to an apoptosis-like cell death, as most of the cells were positive for AnnexinV staining and negative for 7-AAD uptake. In addition, the nuclei of MT103-infected MH-S cells presented typical apoptotic features, such as nuclear condensation and fragmentation (Figure 1B). A similar result was found in THP-1 human macrophages (supplementary Figure 1). Contrariwise to the wild-type strains, the attenuated *M. tuberculosis* MTBVAC was unable to trigger apoptosis in the MH-S cells.

As mentioned earlier, we previously showed that BCG vaccine strain is unable to induce apoptosis (Aporta et al, 2012). In order to understand this phenotype, in the present work we infected MH-S cells with recombinant BCG strains complemented with selected RD regions absent from the genome of BCG, i.e. RD1, RD4, RD5, RD7, (Gordon et al, 1999), and subsequently we analyzed the ability of these strains to trigger apoptosis. Our results clearly indicate that only the BCG::RD1 strain, which has a reconstituted ESX-1secretion system, recovered the ability to induce apoptosis. BCG strains complemented with RD4, RD5 or RD7

behaved like the parental BCG Pasteur control strain (Figure 1C). Of note, the initial percentage of infected cells was similar for all tested strains, as analysed by using GFP expressing strains (data not shown), which makes it unlikely that the observed differences in results were due to possible variability in the infectious bacterial load.

ESAT-6, which is secreted through the ESX-1 system, has been shown to cause apoptosis on host cells (Choi et al, 2010). Thus, we corroborated that ESAT-6 was secreted in cell culture supernatants only by the apoptosis-inducing strains, as shown in Figure 1D. As a control we used antigen Ag85A, which is secreted via the general SecA-dependent secretion pathway. Presence of the Ag85A in the supernatants of MTBVAC and BCG cultures confirmed that the absence of ESAT-6 secretion in these strains was not due to differences in the quality of bacterial cultures.

Similarly to BCG and MTBVAC, we observed that *M. tuberculosis* H37Ra, an attenuated version of *M. tuberculosis* H37 strain, did not trigger apoptosis (Supplementary Figure 2). Remarkably, *M. tuberculosis* H37Ra does not secrete ESAT-6 due to a point mutation in the DNA binding region of the *phoP* gene (Frigui et al, 2008; Wang et al, 2007).

APOPTOSIS IN VIVO IS INDUCED BY ESAT-6 SECRETING MYCOBACTERIA

To investigate the physiological relevance of our findings obtained with cultured cells, we extended our studies to an *in vivo* infection model, using C57BL/6 mice. To this aim, we intranasally challenged mice with *M. tuberculosis* MT103, MTBVAC, BCG or BCG::RD1 strains and measured the bacterial burden four weeks post-challenge. Replication of bacteria was only observed in MT103- and BCG::RD1-infected animals, whereas attenuated MTBVAC and BCG strains were unable to grow in lungs (Figure 2A). Differences in replication between virulent and attenuated strains were also substantial in spleen (supplementary Figure 3). Consequently, Ziehl-Neelsen staining revealed the presence of mycobacteria only in the lungs of mice exposed to MT103 or BCG::RD1 strains (supplementary Figure 4A). In parallel, histopathology analyses also revealed striking differences between the organs of animals from the different sets of groups. In the case of MTBVAC- or BCG groups the appearance of the lungs was similar to the non-infected controls. Lungs from mice infected with MT103 or

BCG::RD1 strains presented wide areas of consolidation and inflammation with a high degree of cellular infiltration (Figure 2B). F4/80 staining confirmed that most of the infiltrated cells were macrophages (supplementary Figure 4B). In order to analyse whether bacterial replication correlated with apoptosis in the infected tissues we analyzed the presence of active-caspase 3 in the lungs. As shown in Figure 2C, only lungs infected with MT103 or BCG::RD1 presented a high level of caspase-3 activation. Remarkably, presence of apoptotic cells was mainly restricted to the areas of tissue consolidation and inflammation in the lung tissues of these two groups. Caspase-3 activation was practically absent in the lungs of mice inoculated with BCG or MTBVAC (Figure 2C). Altogether, our data indicate that apoptosis induced *in vivo* by mycobacteria correlates with the presence of a functional ESX-1 system and secretion of ESAT-6, suggesting that ESAT-6 also plays a pro-apoptotic role under physiological conditions in mouse model.

APOPTOSIS INDUCTION CORRELATES WITH CELL-TO-CELL BACTERIAL SPREAD CAPACITY IN VITRO

Previous reports from different research groups suggest that ESX-1 systems in *M. tuberculosis* and in the closely related *Mycobacterium marinum* are essential for cell-to-cell spread of bacteria (Davis & Ramakrishnan, 2009; Gao et al, 2004; Guinn et al, 2004). In good agreement with these observations, we here show that only the ESAT-6 secreting strains MT103 and BCG::RD1, but not the attenuated MTBVAC and BCG, replicated within MH-S macrophages (Figure 3A). Nevertheless, such replication assays do not discern whether replication occurs only in the cells initially infected, or if the bacteria are able to colonize new, yet uninfected cells over time. To tackle this question in more detail, we used GFP-expressing mycobacterial strains to monitor macrophage infection at the single cell level (Valdivia et al, 1996). As shown in Figure 3B, the percentage of initially infected (GFP positive) host cells only increased when apoptosis-inducing MT103 and BCG::RD1 strains were used. This result indicated that these bacteria were spreading into new host cells that had not been initially infected. Conversely, the percentage of GFP-positive cells did not change in cell cultures infected with MTBVAC or BCG even though the initial percentage of GFP-positive cells was similar to that seen for

virulent strains, strongly suggesting that non-virulent strains are unable to spread into new host cells due to efficient host control mechanisms. These findings further suggest that apoptosis might be a mechanism that efficiently contributes to host colonization by pathogenic *M. tuberculosis*.

APOPTOSIS MODULATION IN VITRO *ALTERS CAPACITY OF MYCOBACTERIA TO INFECT NEW CELLS*

To assess whether apoptosis affects cell-to-cell bacterial propagation, we induced or inhibited apoptosis on host cells and we monitored potential variations in the capacity of bacteria to infect new cells using the GFP-expressing strains.

Since BCG, which does not trigger apoptosis in macrophages, was unable to spread to new cells, we wondered whether we could revert this phenomenon if we externally induced apoptosis following infection. We incubated MH-S macrophages with BCG in presence of increasing concentrations of staurosporine, a potent apoptosis-inducing drug. Previously, we corroborated that staurosporine was killing cells by apoptosis, which is demonstrated by the predominant AnnexinV+7AAD- phenotype observed after overnight staurosporine incubation (supplementary Figure 5). As shown in Figure 4A, under staurosporine treatment, the increasing percentage of dead cells (AnnexinV+7AAD-) significantly correlated with the percentage of GFP-positive cells infected with BCG. Hence, in the presence of apoptosis, attenuated mycobacteria, which are normally unable to colonize new cells, do gain the capacity to spread.

To corroborate these data, we used the contrary approach: we inhibited apoptosis and tested the capacity of virulent *M. tuberculosis* to spread from cell to cell. It was previously shown that activation of p38MAPK leads to apoptosis of *M. tuberculosis*-infected macrophages (Kundu et al, 2009) as well as neutrophils (Aleman et al, 2004). To further test the possible role of apoptosis for bacterial propagation, we monitored MT103 infection in the presence of SB202190, a specific p38MAPK inhibitor. Presence of the inhibitor at a concentration of 10 μM clearly abrogated MT103-induced apoptosis. More importantly, inhibition of macrophage apoptosis dramatically abrogated *M. tuberculosis* colonization of new non-infected cells (Figure 4B). This finding corroborates that apoptosis is a much more important mechanism for *M.*

tuberculosis-infection than previously thought. Percentage of infected cells at 0h was similar in presence or absence of the inhibitor, indicating that SB202190 was not intrinsically affecting bacterial infection capacity. In addition, we also measured the influence of SB202190 on bacterial replication. MT103 intracellular replication was partially impaired in the presence of the inhibitor (Figure 4B). Nevertheless, replication seemed to be much less affected than bacterial spread, indicating that bacterial propagation and replication are not totally dependent processes. Dissociation of these two parameters has been already described by other authors (Guinn et al, 2004).

DISCUSSION

In this work, we analyzed the pro-apoptotic capacity of several virulent and attenuated mycobacterial strains in MH-S cells, a validated model of immortalized murine alveolar macrophages that mimics very closely the characteristics of primary alveolar macrophages in the interaction with mycobacteria (Melo & Stokes, 2000). In combination with our previous results (Aporta et al, 2012), we here show that induction of host-cell apoptosis is a common feature of virulent *M. tuberculosis* strains that is apparently lost by attenuated strains with impaired ESX-1 secretion system. We have seen that virulent *M. tuberculosis* strains like MT103, H37Rv, or members of the Beijing-family as well as RD1-complemented BCG::RD1 strains are able to replicate in macrophages both under *in vitro* and *in vivo* conditions, thereby inducing high levels of apoptosis. Conversely, attenuated strains like BCG, H37Ra or the live attenuated *M. tuberculosis*-based vaccine candidate MTBVAC are practically unable to kill host cells. In addition, *in vivo* data obtained in this work validate these observations under physiological conditions. Our results also indicate that the ability to induce apoptosis in host cells is independent of the family origin of the *M. tuberculosis* strains, as no significant differences among Beijing strains and MT103 or H37Rv strains were found.

The subject of whether or not mycobacteria can induce apoptosis in host cells is widely discussed in the scientific literature. However, the observations and interpretation of data in different experimental settings seem to be rather heterogenic. While our data, in agreement with some recent reports, suggest that only virulent *M. tuberculosis* strains induce apoptosis on host cells (Derrick & Morris, 2007; Grover & Izzo, 2012; Lim et al, 2011), there is also evidence from other studies (Briken & Miller, 2008; Chen et al, 2006; Gan et al, 2008; Sly et al, 2003) that apoptosis is triggered *in vitro* preferentially by attenuated strains and that virulent strains showed more tendency to inhibit apoptosis in host macrophages rather than promoting it (Behar et al, 2011; Martin et al, 2012).Use of different *in vitro* experimental models, cell lines or protocols could provide some explanation for such discrepancies. However, most of the *in vivo* evidences, including ours, strongly indicate that apoptosis occurs during virulent *M. tuberculosis* infection. Apoptotic markers such as active-caspase 3 or TUNEL have been found

in infected human and mouse lungs (Aporta et al, 2012; Keane et al, 1997; Seimon et al, 2010). Remarkably, *in vivo* data indicate that apoptosis induced by *M. tuberculosis* in lungs is preferentially restricted to granulomatous lesions. This would support the idea that apoptosis is an infectious mechanism, as previously described for *M. marinum* during zebra fish infection, which causes apoptosis in an ESX-1 dependent mechanism, to attract and infect fresh macrophages, thereby generating secondary granuloma (Davis & Ramakrishnan, 2009). In fact, a similar mechanism of infection has been proposed for *M tuberculosis* (Ernst et al, 2007). Supporting these findings, here we show that unlike BCG parental strain, the RD1-complemented BCG::RD1 strain recovers the ability to induce apoptosis in infected lungs *in vivo*. BCG::RD1 restores functional ESX-1 secretion system and has been shown to recover virulence both *in vitro* and *in vivo* (Pym et al, 2002) in agreement with data presented in this work.

To address the role of apoptosis in the dissemination of *M. tuberculosis* we monitored macrophage infection using GFP-expressing strains, observing that percentage of host cells initially infected increased when apoptosis-inducing virulent strains were used to infect. Our interpretation of these results was that virulent bacteria were spreading from cell to cell. Nevertheless, other plausible explanation of these data could be that the most of the cells could be initially infected but below the sensitivity of flow cytometry to detect GFP bacteria in macrophages, and simple growth of the bacteria within the cells could explain the increase of GFP-positive cells. To elucidate this question, we confirmed by fluorescence microscopy, which is able to detect a single bacterium within a cell, that not all the cells were initially infected and the percentage of infection was equivalent to that observed by flow cytometry (data not shown), supporting our hypothesis of that the results observed corresponded to cell-to-cell bacterial spread. Data clearly show that only apoptosis-inducing bacteria are able to colonize new cells that were initially non-infected. In agreement with the mechanism described for *M. marinum* (Davis & Ramakrishnan, 2009), we hypothesize that colonization of new cells occurs by phagocytosis of mycobacteria-containing apoptotic bodies. However, the differences between virulent and attenuated strains do not allow discerning whether apoptosis is a cause, or

on the contrary, just a collateral effect of the infection. To distinguish between these possibilities, we used two strategies: i) we promoted apoptosis in the presence of attenuated BCG and ii) we inhibited pro-apoptotic pathways prior to infection with virulent MT103. Infection follow-up in both scenarios allowed us to establish a link between apoptosis and cell-to-cell propagation by ESAT-6-secreting mycobacteria. BCG spread was favoured when apoptosis was induced in host cells, whereas *M. tuberculosis* was unable to infect new cells if cell death was inhibited. In relation to these findings, Guinn and colleagues described that the *M. tuberculosis* H37RvΔRD1 mutant accumulated in initially infected host cells, but unlike the H37Rv wild-type strain, it was unable to spread to new cells (Guinn et al, 2004). Supporting the role of ESAT-6-induced apoptosis for cell-to-cell bacterial spread, our data suggest that the intracellular phenotype of H37RvΔRD1 could be due to the inability of this strain to induce apoptosis (Derrick & Morris, 2007).

A recently proposed mechanism of virulence is the capacity of *M. tuberculosis* to disrupt phagosome membrane in an ESAT-6-dependent fashion, reaching the cytosol and causing cell death (Houben et al, 2012; Simeone et al, 2012; van der Wel et al, 2007). A clear correlation between contact of bacteria with cytosol and cell death induction was noted, which suggests that *M. tuberculosis* needs to gain access to the cytoplasm to activate p38MAPK signalling cascade leading to host cell death. Finally, it is not clear whether ESAT-6 is involved only in the process of disruption of the phagosomal membrane, or if it also actively participates in triggering cell death, even though data obtained using purified ESAT-6 protein seem to point to it as a pro-apoptotic molecule on its own (Choi et al, 2010).

For an intracellular pathogen, it is logical to speculate that the most successful way to infect the host is to spread from cell to cell without exposing itself to extracellular milieu. In the case of *M. tuberculosis*, multiple mechanisms to prevent intracellular defences have been described, but there is little evidence for other mycobacterial strategies to overcome extracellular antimicrobial barriers. Consistent with what has been observed for other intracellular pathogens, such as *Salmonella* (Guiney, 2005), apoptotic cells may be the perfect Trojan horse for *M. tuberculosis* to colonize fresh macrophages and ensure a safe replication niche.

In vivo replication studies in mice indicate that despite the low number of bacteria used initially to infect, *M. tuberculosis* is able to replicate in the lungs for approximately three weeks without the opposition of an adapted immune response (Cooper, 2009; Wolf et al, 2008). Why this pathogen remains "hidden" from the immune system during this crucial early phase of the infection remains unclear. Apoptosis induction by *M. tuberculosis* could help elucidate such questions. By triggering apoptosis, *M. tuberculosis* could create new niches for intracellular replication preventing exposition to extracellular host defences, and in the absence of the inflammatory reaction associated with necrotic cell death. Additionally, *M. tuberculosis* has been shown to inhibit autophagy in an ESAT-6-dependent manner (Romagnoli et al, 2012). This could contribute to keep bacteria occult, as autophagy has been shown to be an important bactericidal process that leads to pathogen antigen presentation (Gutierrez et al, 2004; Jagannath et al, 2009).

A better understanding of the mechanisms implicated in the dissemination of *M. tuberculosis* from cell-to-cell, which could result important for bacterial escape from the host immune system, should allow the design of new strategies to attenuate mycobacterial strains and to develop new better vaccines that protect against pulmonary tuberculosis.

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Figure 1. Apoptosis on MHS cells is restricted to ESAT6-secreting strains.

A, **C**, MH-S murine macrophages were mock-treated or infected (MOI 10:1) with the indicated strains. 72 hours post-infection, cells were stained with annexinV and 7-AAD and analyzed by flow cytometry. A representative experiment is shown in the right panels. Data in the graphs (left panels) are represented as mean±SD. Three independent experiments were at least performed. Statistical analysis was done with one-way ANOVA and Bonferroni's post-test comparing each strain to non-infected control. Upper symbols = statistical analyses of Ann+AAD+ cells; lower symbols = statistical analyses of Ann+AAD- cells. ns = not statistically significant; *, **, *** = statistically significant; * p<0,05; ** p<0,01; *** p<0,001. **B**, for fluorescence microscopy studies MH-S cells were infected with GFP-expressing MT103 bacteria and stained 72 hours post-infection with Hoechst 33342. A representative image is shown in the figure. **D**, ESAT-6 secretion was analyzed by Western-blot. Log-phase cultures supernatants from the indicated strains were obtained, and 10 μg of total protein per well were loaded for SDS-PAGE. A representative Western blot image is shown.

Figure 2. *In vivo* apoptosis is limited to ESAT6-secreting strains.

Groups of five C57BL/6 mice were intranasally infected with approximately 1000 CFUs/mouse of MT103, MTBVAC, BCG or BCG::RD1 strains. At 28 days post-infection, animals were humanely sacrificed and lungs harvested for *in vivo* studies. **A**, CFUs in lungs were determined. Representative data of two independent studies are shown. Statistical analysis was done with one-way ANOVA and Bonferroni's post-test. *, **, *** = statistically significant; * p<0,05; ** p<0,01; *** p<0,001. **B**, lung histopathology was evaluated by haematoxylin/eosin (HE) staining. **C**, apoptosis incidence was evaluated by immunohistochemical staining with a specific antibody for the active form of the caspase 3. Representative images (10x magnification for HE and 600x for caspase 3 staining) of mock-treated or MT103-, MTBVAC-, BCG- and BCG::RD1- infected lungs are shown.

Figure 3. Intracellular replication and cell-to-cell bacterial spread correlates with capacity to induce apoptosis.

MH-S murine macrophages were mock-treated or infected with the indicated GFP-expressing strains at the described MOIs. **A**, at 0 and 72 hours post-infection (MOI 5:1), cells were lysed and bacterial burden counted. A representative experiment of two independent studies is shown. **B**, at the indicated times post-infection, percentage of GFP-positive cells was determined by flow cytometry. Representative dot-plot diagrams are shown in the upper panels. Data in the graphs (lower pannels) are represented as mean±SD. Two independent experiments were performed. Statistical analysis was done with two-way ANOVA and Bonferroni's post-test. ns = not statistically significant; *, **, *** = statistically significant; * p<0,05; ** p<0,01; *** p<0,001.

Figure 4. Apoptosis modulation alters cell-to-cell bacterial spread.

MH-S murine macrophages were mock-treated or infected with 5 to 10 bacteria per cell of the indicated GFP-expressing strains. **A**, BCG-infected cells were incubated for 20 hours with increasing concentrations of staurosporine (up to 0.5 µM), and percentage of GFP-positive cells was evaluated by flow cytometry. Representative dot-plot diagrams are shown in the upper panels. Percentage of GFP-positive cells determined at each concentration of staurosporine was represented against percentage of apoptotic cells (AnnV+AAD- cells). A representative graph of two independent experiments demonstrating significant positive correlation between both parameters is shown in the lower graph. **B**, MH-S cells were infected with MT103 in presence of the indicated concentrations of the SB202190 inhibitor. 72 hours post infection, apoptosis determined by AnnexinV and 7-AAD staining (left graph), percentage of GFP-positive cells (right upper graph) and bacterial burden (right lower graph) were evaluated. Data in the graphs are represented as mean±SD. Two independent experiments were at least performed. Statistical analysis was done with one-way ANOVA (left panel), two-way ANOVA (right upper panel), both with Bonferroni's post-test, or with t-student test (right lower graph) *, **, *** = statistically significant; * p<0,05; ** p<0,01; *** p<0,001.

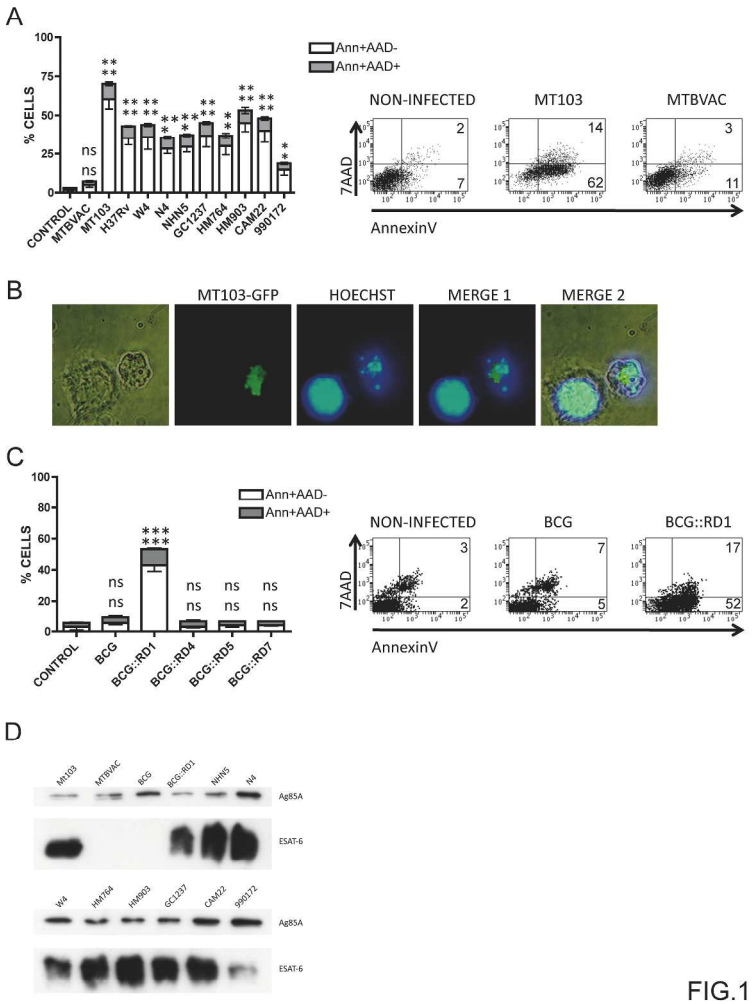

FIG.1

297x420mm (300 x 300 DPI)

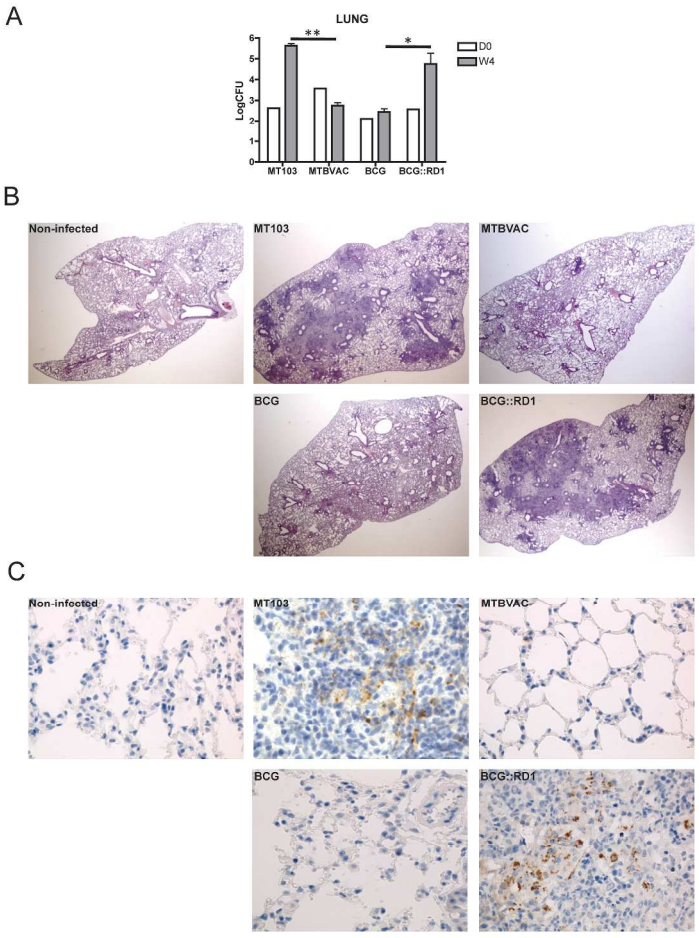

FIG.2

297x421mm (300 x 300 DPI)

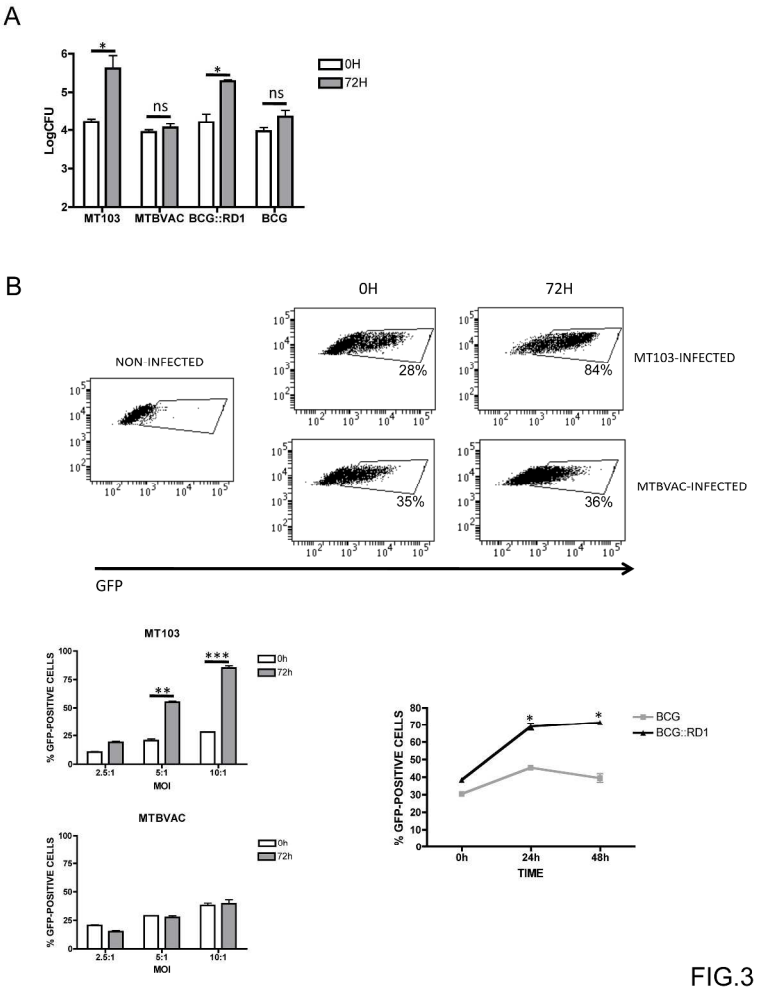

FIG.3

297x421mm (300 x 300 DPI)

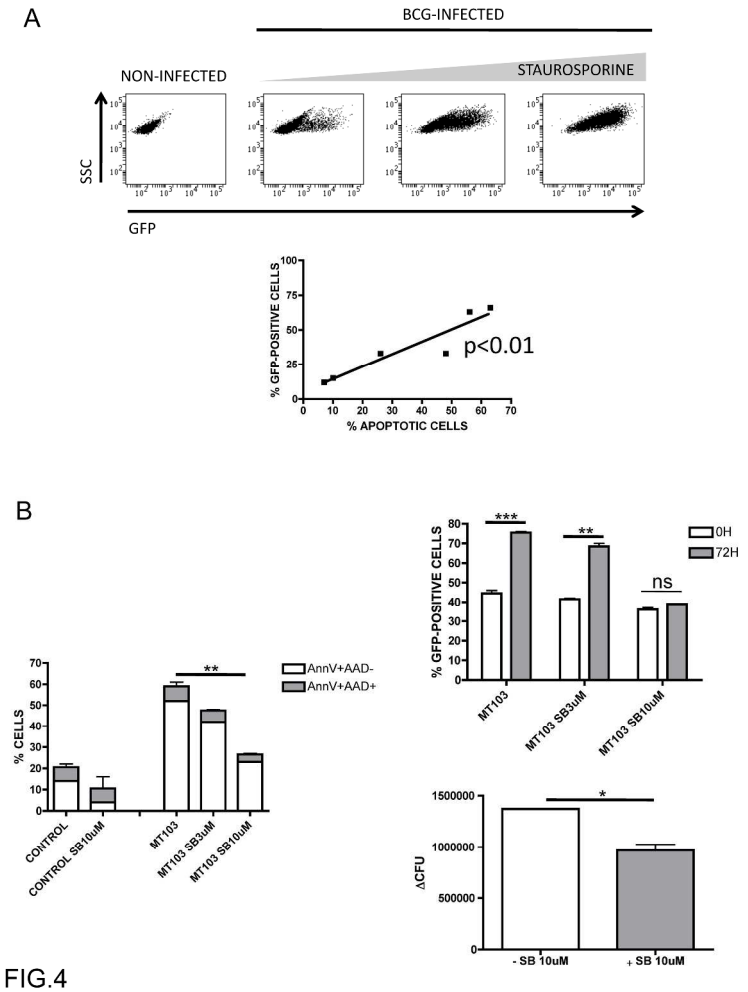

FIG.4

297x421mm (300 x 300 DPI)