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Bim is a crucial regulator of apoptosis induced by *Mycobacterium tuberculosis*

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***Mycobacterium tuberculosis*, the causative agent of tuberculosis, induces apoptosis in infected macrophages *in vitro* and *in vivo*. However, the molecular mechanism controlling this process is not known. In order to study the involvement of the mitochondrial apoptotic pathway in *M. tuberculosis*-induced apoptosis, we analysed cell death in *M. tuberculosis*-infected embryonic fibroblasts (MEFs) derived from different knockout mice for genes involved in this route. We found that apoptosis induced by *M. tuberculosis* is abrogated in the absence of Bak and Bax, caspase 9 or the executioner caspases 3 and 7. Notably, we show that MEF deficient in the BH3-only BCL-2-interacting mediator of cell death (Bim) protein were also resistant to this process. The relevance of these results has been confirmed in the mouse macrophage cell line J774, where cell transfection with siRNA targeting Bim impaired apoptosis induced by virulent mycobacteria. Notably, only infection with a virulent strain, but not with attenuated ESX-1-defective strains, such as Bacillus Calmette-Guerin and live-attenuated *M. tuberculosis* vaccine strain MTBVAC, induced Bim upregulation and apoptosis, probably implicating virulence factor early secreted antigenic target 6-kDa protein in this process. Our results suggest that Bim upregulation and apoptosis is mediated by the p38MAPK-dependent pathway. Our findings show that Bim is a master regulator of apoptosis induced by *M. tuberculosis*.**
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Mycobacterium tuberculosis, the causative agent of tuberculosis, is primarily an intracellular pathogen that has successfully developed strategies to colonise host alveolar macrophages and overcome their bactericidal defence mechanisms.[1]

Apoptosis is a physiological type of cell death characterised by the preservation of the plasma membrane integrity, which prevents local inflammatory reactions and tissue damage. Intracellular pathogens have co-evolved with the host to develop strategies for modulation of host cell apoptosis to favour infection.[2] During *M. tuberculosis* infection, presence of apoptotic cells has been detected in lungs from both infected humans and mice.[3–5] ESX-1 secretion system, which regulates early secreted antigenic target 6-kDa protein (ESAT-6) secretion, seems to play a crucial role in apoptosis induction and virulence during mycobacterial infection.[3,6] It has been shown that attenuated strains, like Bacillus Calmette-Guerin (BCG) and the live-attenuated *M. tuberculosis* vaccine *Mycobacterium tuberculosis* vaccine strain (MTBVAC),[7] which lack a functional ESX-1 secretion system, have lost their ability to induce apoptosis and cell death.[3,8] Altogether, these results suggest that the ability to induce apoptotic cell death is a feature characteristic of virulent strains. Indeed, similarly to other authors, we have shown that apoptosis triggered by virulent mycobacteria is required for bacterial spread.[3,9]

The activation of the mitochondrial cell death pathway is regulated by the Bcl-2 family of proteins consisting of pro-apoptotic (Bak, Bax, Bim, Bid and so on) and anti-apoptotic (Bcl-2, Bcl-X_L, Mcl-1 and so on) members, whose activity is reciprocally modulated.[10] BH3-only pro-apoptotic proteins (i.e., Bid, BCL-2-interacting mediator of cell death (Bim), Puma and Noxa) interfere with anti-apoptotic proteins Bcl-2, Bcl-X_L or Mcl-1, and induce Bak and Bax activation by conformational change, leading to mitochondrial permeabilization.[11] Pore formation on mitochondrial membrane leads to the release of pro-apoptotic factors to cytosol. One of these molecules, cytochrome *c*, is necessary to activate caspase 9,[12] which activates the effector caspases 3 and 7 by cleavage. These are ultimately responsible for the appearance of the apoptotic phenotype.

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Abbreviations: MEF, mouse embryonic fibroblast; Wt, wild type; DKO, double knockout; MTBVAC, *Mycobacterium tuberculosis* vaccine strain; ESAT-6, early secreted antigenic target 6-kDa protein; 7-AAD, 7-actinomycin D; annV, annexin V; PS, phosphatidylserine; Bim, BCL-2-interacting mediator of cell death; ER, endoplasmic reticulum; BCG, Bacillus Calmette-Guerin

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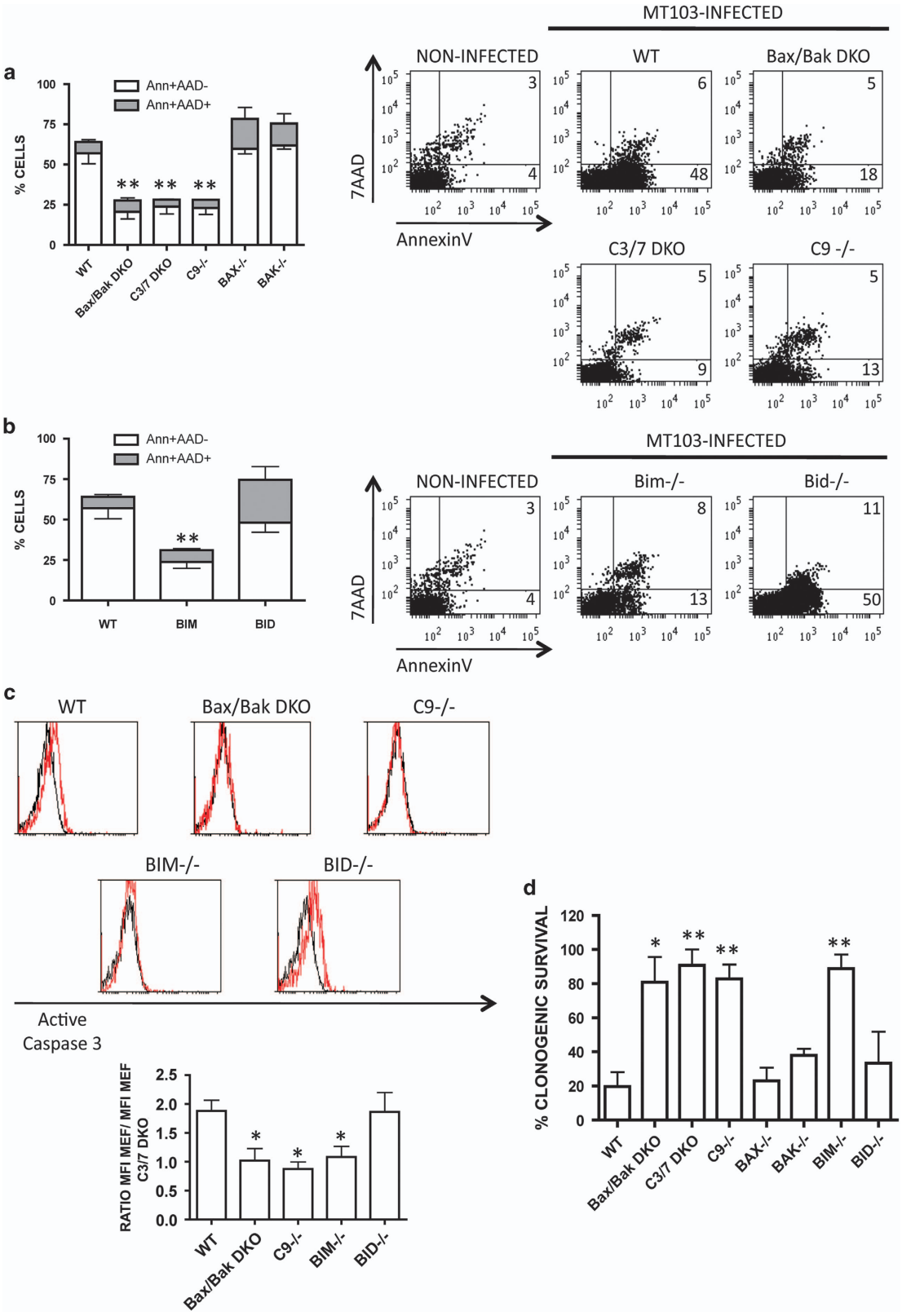
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The intracellular mediators of apoptosis induced by *M. tuberculosis* are poorly understood. Previous works have shown that virulent *M. tuberculosis* strains are able to activate the mitochondrial cell death pathway including cytochrome *c* release and caspase activation.[4,13] However, the molecular mechanism including the involvement of the Bcl-2 family in this process remains unknown. In this work, we conducted an in-depth analysis of the implication of different pro-apoptotic members of the Bcl-2 family during apoptosis induced by the clinical isolate MT103 in different cell lines. We have identified the BH3-only protein Bim as a key modulator of apoptosis induction and bacterial spread.

Results

M. tuberculosis **induces apoptosis through the mitochondrial cell death pathway.** It has been previously described that the mitochondrial apoptotic pathway is activated in *M. tuberculosis*-infected macrophages.[14] However, the role of this signalling route has not been studied in detail and the molecular players involved are still elusive. Thus, we used MEF cells from mice knockout for different proteins of the intrinsic cell death pathway in order to dissect the molecular mechanism of apoptosis induced by *M. tuberculosis*. MEF cells were infected with the virulent *M. tuberculosis* clinical isolate MT103, and apoptosis was analysed by monitoring phosphatidylserine (PS) translocation and membrane integrity. We analysed apoptosis at day 7 post infection because at this time point we observed the highest rate of apoptotic cells (Supplementary Figure S1). As shown in Figure 1a, wild-type MEF (MEF.wt) cells showed a characteristic apoptotic-like phenotype, staining with Annexin V and maintaining cellular impermeability to 7-actinomycin D (7-AAD). In contrast, MEF deficient for Bax and Bak (MEF.Bak/Bax DKO), caspase 9 (MEF.Casp9 $-/-$), or the executioner caspases 3 and 7 (MEF.Casp3/7 DKO) were profoundly resistant to MT103-induced apoptosis. Single Bak- or Bax-deficient MEF cells were as susceptible to apoptosis as MEF.wt (Figure 1a), indicating that presence of either Bak or Bax is sufficient to activate the mitochondrial cell death pathway during MT103 infection. Results obtained with MEF.Casp9 $-/-$ and MEF.Casp3/7 DKO cells confirmed the implication of the mitochondrial apoptotic route. Both cell lines were resistant to apoptosis, indicating that MT103 activates the classical mitochondrial route including the activation of caspase 9 and the executioner caspases 3 and 7. We also noticed a residual cell death of about 25% in all MEF-resistant cell lines, suggesting that MT103 may exert some cytotoxicity in host cells in a mitochondria- and caspases 3/7-independent manner.

Apoptosis induced by MT103 in MEF cells is regulated by the BH3-only protein Bim. We studied the possible role of the BH3-only proteins, Bim and Bid, as activators of the intrinsic route in MT103-infected MEF cells. Bid has been identified as the BH3-only protein that links the extrinsic and the intrinsic apoptotic pathways.[15] Bim has being identified to respond to cellular stress stimuli, being a key regulator of apoptosis induced by endoplasmic reticulum (ER) stress response.[16] As shown in Figure 1b, Bid-deficient (MEF.Bid $-/-$) cells underwent apoptosis when infected with MT103, suggesting that this BH3-only protein is not triggering the mitochondrial route in our model. On the contrary, MEF.Bim $-/-$ cells were completely resistant to apoptosis, similar to MEF.Bax/Bak DKO, MEF.Casp9 $-/-$ and MEF.Casp3/7 DKO cells. These data indicate that Bim could result important for *M. tuberculosis*-induced apoptosis, at least in MEF cells.

Next, we analysed caspase 3 activation by flow cytometry. As shown in Figure 1c, active caspase 3 was only detected in MEF.wt and MEF.Bid $-/-$ cells infected with MT103. Fluorescence intensity in MT103-infected MEF.Bax/Bak DKO, MEF.Casp9 $-/-$ or MEF.Bim $-/-$ cells was similar to MEF.Casp3/7 DKO-negative controls. This result corroborates that caspase 3 activation occurs downstream of the mitochondria.

Bim deficiency recovers the clonogeneic potential of MEF cells infected with virulent MT103 strain. Results above indicated that *M. tuberculosis* could not induce cell death in Bax/Bak, Bim, Casp9 or Casp3/7 knockout MEF cells up to 7 days post infection. In order to elucidate whether cell death would take place at later time points, we performed a clonogenic assay. This kind of assays represents a gold standard test in the field of cell death research used to discern if a specific stimulus is able to kill a target cell at long term, and is based on the ability of treated cells to proliferate and to form colonies. Our previous work showed that *M. tuberculosis*-infected MEF cells lose clonogenic capacity.[4] Clonogenic assay studies performed in this work showed that MEF.Bax/Bak DKO, MEF.Casp9 $-/-$, MEF.Bim $-/-$ or MEF.Casp3/7 DKO cells were totally resistant to *M. tuberculosis* cytotoxic effect, whereas MEF.Bid $-/-$ or single MEF.Bax $-/-$ and MEF.Bak $-/-$ cells were as susceptible as MEF.wt to cell death induced by MT103 strain (Figure 1d).

Bim is upregulated in ***M. tuberculosis*****-infected macrophages.** Our results strongly point to Bim as a key factor for triggering apoptosis in infected cells. To assess the possible physiological relevance of this finding,

Figure 1 MT103 induces apoptosis on MEF by activation of the mitochondrial apoptotic route. Wild-type MEF (WT) and MEF knockouts for Bax, Bak, caspases 3 and 7(C3/7 DKO), caspase 9 (C9), Bak and Bax (Bax/Bak DKO), Bim, Bid were infected with MT103 (MOI 30 : 1) during seven days. (**a** and **b**) Cells were stained with annexinV and 7-AAD, and analysed by flow cytometry. Representative dot plots are shown in the right panels. Data in the graphs (left panels) are represented as mean ± S.E.M. of four independent experiments. (**c**) Cells were stained with an anti-active caspase 3 antibody and analysed by flow cytometry. Representative histograms are shown in the upper panel. Black and red lines represent infected-C3/7 DKO MEF and the indicated MEF types, respectively. Data in the graph (lower panel) correspond with the ratio of mean fluorescent intensities (MFI) of the different infected MEF types *versus* the C3/7 DKO MEF control and are shown as mean ± S.E.M. of three independent experiments. (**d**) Seven days post infection, 150 cells per well were seeded and incubated in fresh medium during eight additional days. Survival was calculated as percentage of colonies relative to the number of colonies in the non-infected controls. Data in the graphs are represented as mean ± S.E.M. of two independent experiments. Statistical analysis was done using one-way ANOVA and Bonferroni's *post hoc* test comparing with MT103-infected wild-type cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

we studied the implication of Bim during *M. tuberculosis*-induced apoptosis in macrophages, the main target cell for this pathogen.

We analysed whether Bim expression was altered by *M. tuberculosis* infection in the murine macrophage cell line J774. As shown in Figure 2, Bim appeared strongly upregulated in infected cells, both at transcriptional (Figure 2a) and at protein (Figure 2b) levels. Remarkably, the three main Bim isoforms, BimEL, BimL and BimS,[17] were augmented in infected cells (Figure 2b).

Bim upregulation in macrophages is induced only by virulent *M. tuberculosis* and correlates with apoptosis induction. Our and other authors' works have previously described that ESX-1-deficient strains have lost the ability to induce apoptosis resulting in an attenuated phenotype,[3,9,18,19] as confirmed in MEF cells infected with attenuated strains BCG or MTBVAC (Supplementary Figure S2). In the present study, we analysed whether ESX-1-deficient strains failed to upregulate Bim in infected J774 cells. As shown in Figures 3a and b, attenuated MTBVAC

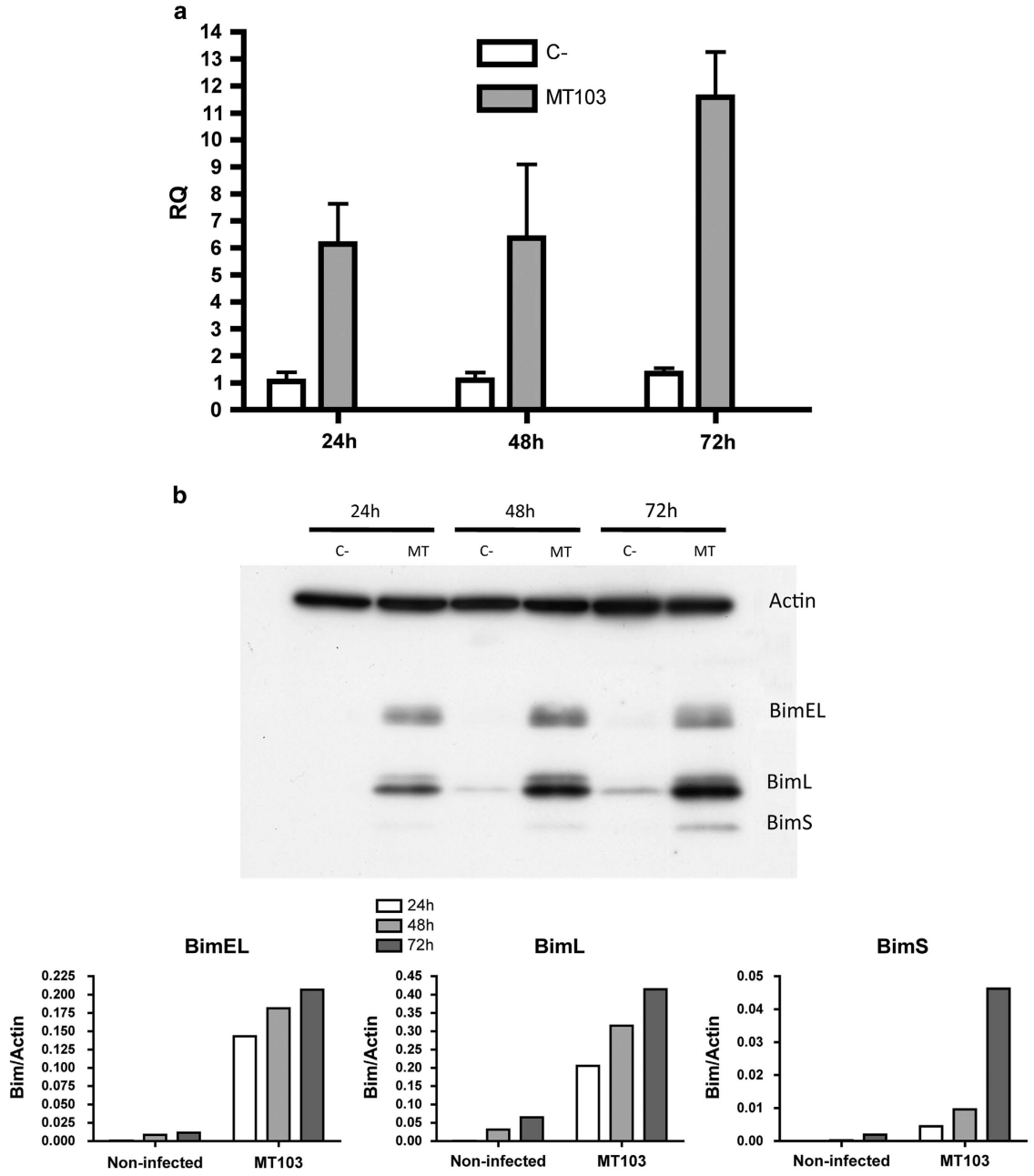

Figure 2 MT103 upregulates Bim in J774 cells. J774 cells were infected with MT103 (MOI 10 : 1) up to 72 h. Bim expression was analysed by RT-qPCR (**a**), or by western blot (**b**) at 24 h, 48 h and 72 h post infection. (**a**) Data in the graph are represented as mean ± S.E.M. of three independent experiments. (**b**) Quantification of the western blot is represented as the ratio of Bim/Actin densities. A representative blot of three independent is shown in the figure

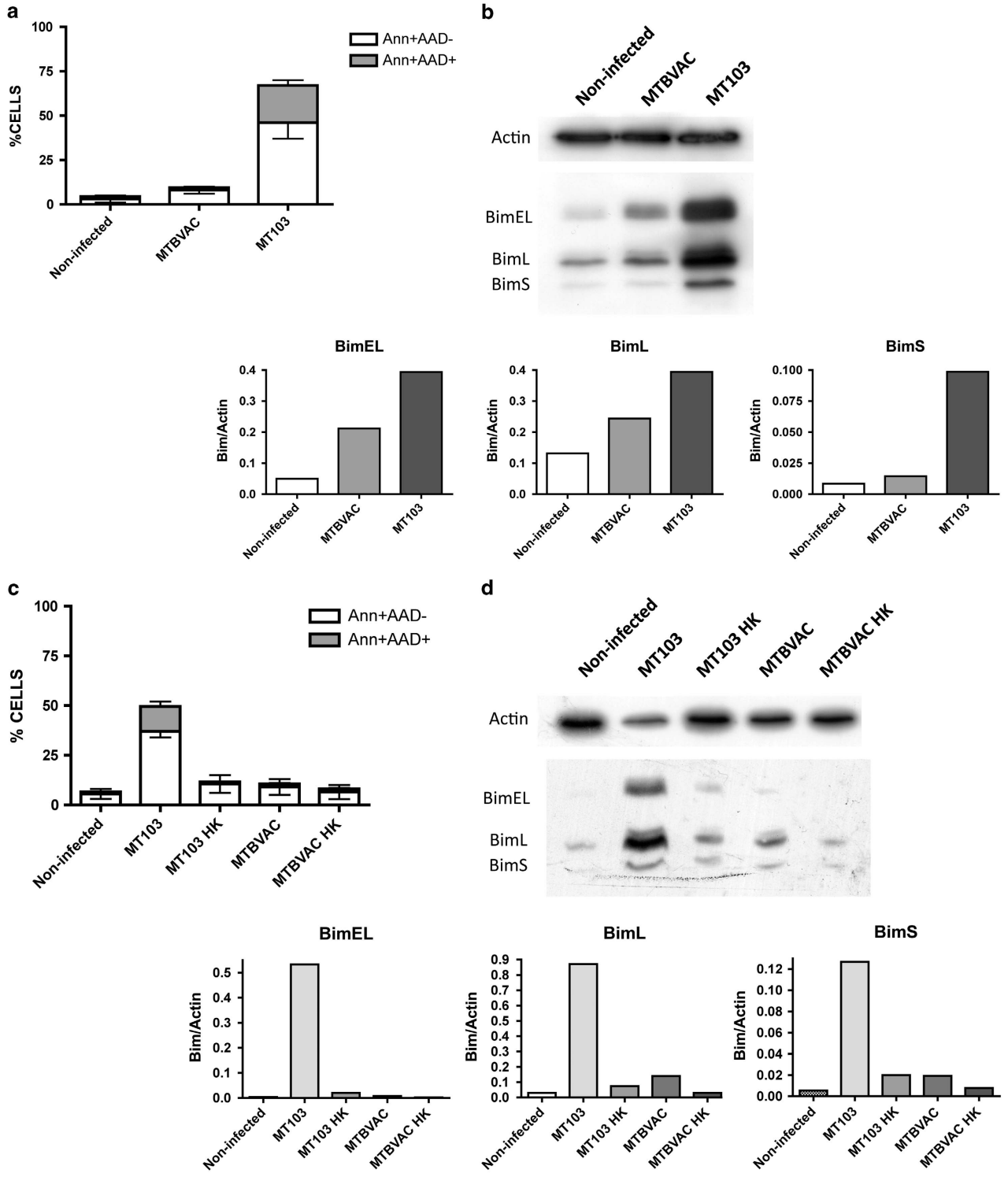

Figure 3 Bim expression is induced by virulent *M. tuberculosis*. (**a** and **b**) J774 cells were infected with MT103 or the live-attenuated vaccine candidate MTBVAC (MOI 10 : 1) for 72 h. (**a**) Cells were stained with annexinV and 7-AAD, and analysed by flow cytometry. Data in the graph are represented as mean ± S.E.M. of three independent experiments. (**b**) A representative blot of two independent experiments is shown. Quantification of the western blot is represented as the ratio of Bim/Actin densities. (**c** and **d**) J774 cells were infected with live or heat-killed (HK) MT103 or MTBVAC bacteria. (**c**) Cells were stained with annexinV and 7-AAD, and analysed by flow cytometry. Data represent the mean ± S.E.M. of two independent experiments. (**d**) A representative blot of two independent experiments is shown. Quantification of the western blot is represented as the ratio of Bim/Actin densities

presented impaired capacity to induce Bim expression as well as to trigger apoptosis in infected cells. MTBVAC principally failed to induce the shorter Bim isoform, BimS, whereas BimEL and BimL isoforms were upregulated when compared with non-infected controls, although to a lesser extent than in cells infected with MT103. In agreement with these data, capacity of MT103 to kill host cells and to induce Bim was abolished when we infected with heat-killed MT103, comparably as observed with non-virulent live MTBVAC. This result strongly implies that *M. tuberculosis* has developed active mechanisms to trigger the pro-apoptotic Bim cascade that leads to host cell death.

Additionally, direct implication of ESX-1 in this process was confirmed infecting J774 cells with BCG and BCG:RD1 strains. BCG:RD1 contains a functional ESX-1 system, which restores both virulence[6] and pro-apoptotic capacity.[3] Comparing both strains, we assessed a strong correlation between apoptosis induction and Bim level expression (Supplementary Figure S3). Finally, we studied the involvement of *phoP* virulence factor in Bim upregulation. As shown in Supplementary Figure S4, *phoP*-deficient strain SO2[20] failed to increase Bim expression in infected J774 cells. Remarkably, complementation of SO2 strain with a functional copy of *phoP* restored the wild-type phenotype.

Bim is involved in *M. tuberculosis*-induced apoptosis in macrophages. Our results show a correlation between Bim upregulation and apoptosis induction during *M. tuberculosis* infection of J774 cells, suggesting a role for Bim in this process. To test whether Bim is required for MT103-induced cell death, we transfected J774 cells with four different siRNA specific for Bim, and subsequently infected cells with MT103. First, we tested the capacity of four different siRNAs to downregulate Bim levels (Figure 4a). The different siRNAs showed variable efficiency to modulate Bim expression when compared with control cells, with siRNA4 resulting the least effective of the four. Importantly, MT103-induced apoptosis was significantly prevented by siRNA1, siRNA2 and siRNA3, implicating Bim in the pro-apoptotic mechanism triggered by *M. tuberculosis* in these cells (Figure 4b). Confirming the specificity of the siRNA approach, siRNA4 did not protect infected cells, which correlated with its limited capacity to downmodulate Bim.

We have previously described that apoptosis inhibition impaired the capacity of *M. tuberculosis* to spread cell to cell and colonise fresh macrophages.[3] Thus, using a GFP-expressing MT103 strain to monitor host cell infection, we observed that infection at 72 h was less efficient in cells transfected with siRNA1, siRNA2 and siRNA3, as compared with non-transfected controls. Importantly, MT103 cell-to-cell spread was not prevented in siRNA4-transfected cells (Figure 4c).

p38MAPK activation mediates *M. tuberculosis*-induced Bim upregulation. In agreement with previous works,[21,22] we recently demonstrated that chemical inhibition of p38MAPK resulted in abrogation of *M. tuberculosis*-induced apoptosis.[3] Consequently, we hypothesised that p38MAPK could be involved in *M. tuberculosis*-induced Bim upregulation.

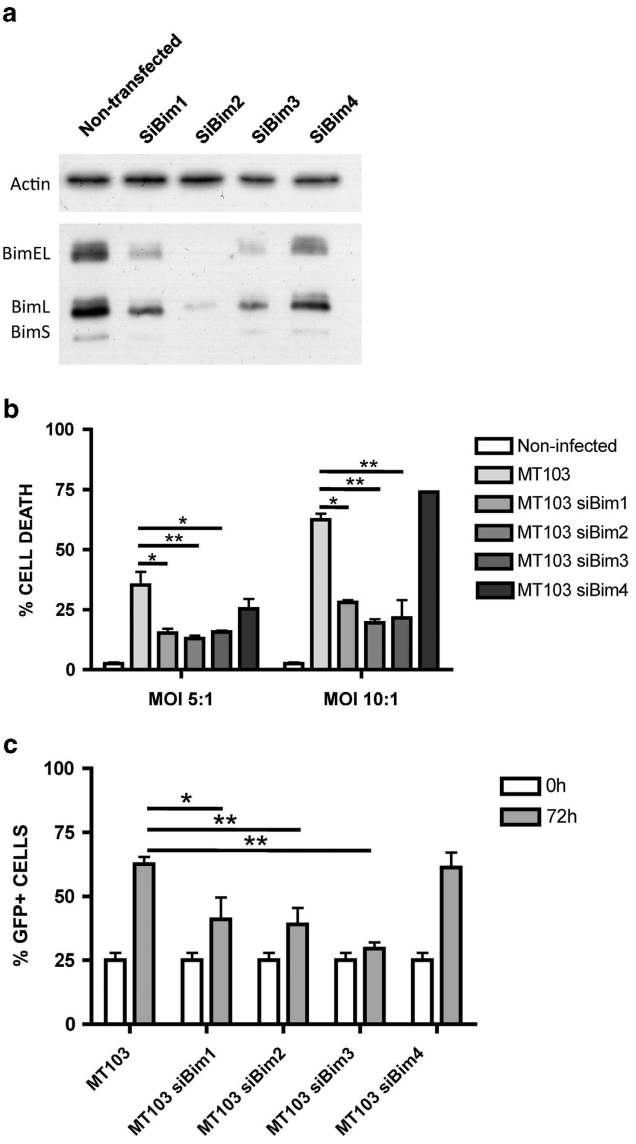

Figure 4 siRNAs targeting Bim impair MT103-induced apoptosis. (**a**) Bim knockdown induced by specific siRNAs was confirmed by western blot. (**b** and **c**) Non-transfected and Bim-specific siRNAs-transfected J774 cells were infected with MT103 (MOI 5 : 1 and 10 : 1) during 72 h. (**b**) Cells were stained with annexinV and 7-AAD, and analysed by flow cytometry. Data in the graphs from four independent experiments are represented as mean ± S.E.M. of the percentage of dead cells. (**c**) Percentage of GFP-positive cells at 0 and 72 h post infection was determined by flow cytometry. Data are shown as mean ± S.E.M. of three independent experiments. Statistical analysis was done using one-way ANOVA and Bonferroni's *post hoc* test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

First, we analysed whether p38MAPK was activated in J774 cells infected with MT103 virulent strain. As shown in Figure 5a, MT103 triggered p38MAPK phosphorylation mainly at early time points (4 h) post infection. Upon infection with MTBVAC, p38MAPK activation at 4 h resulted much less pronounced than observed with the virulent mycobacteria. Looking at later time points (72 h) post infection, we detected that only MT103-infected cells showed sustained p38MAPK activation. Next, we confirmed that the specific p38MAPK inhibitor SB203580 impaired

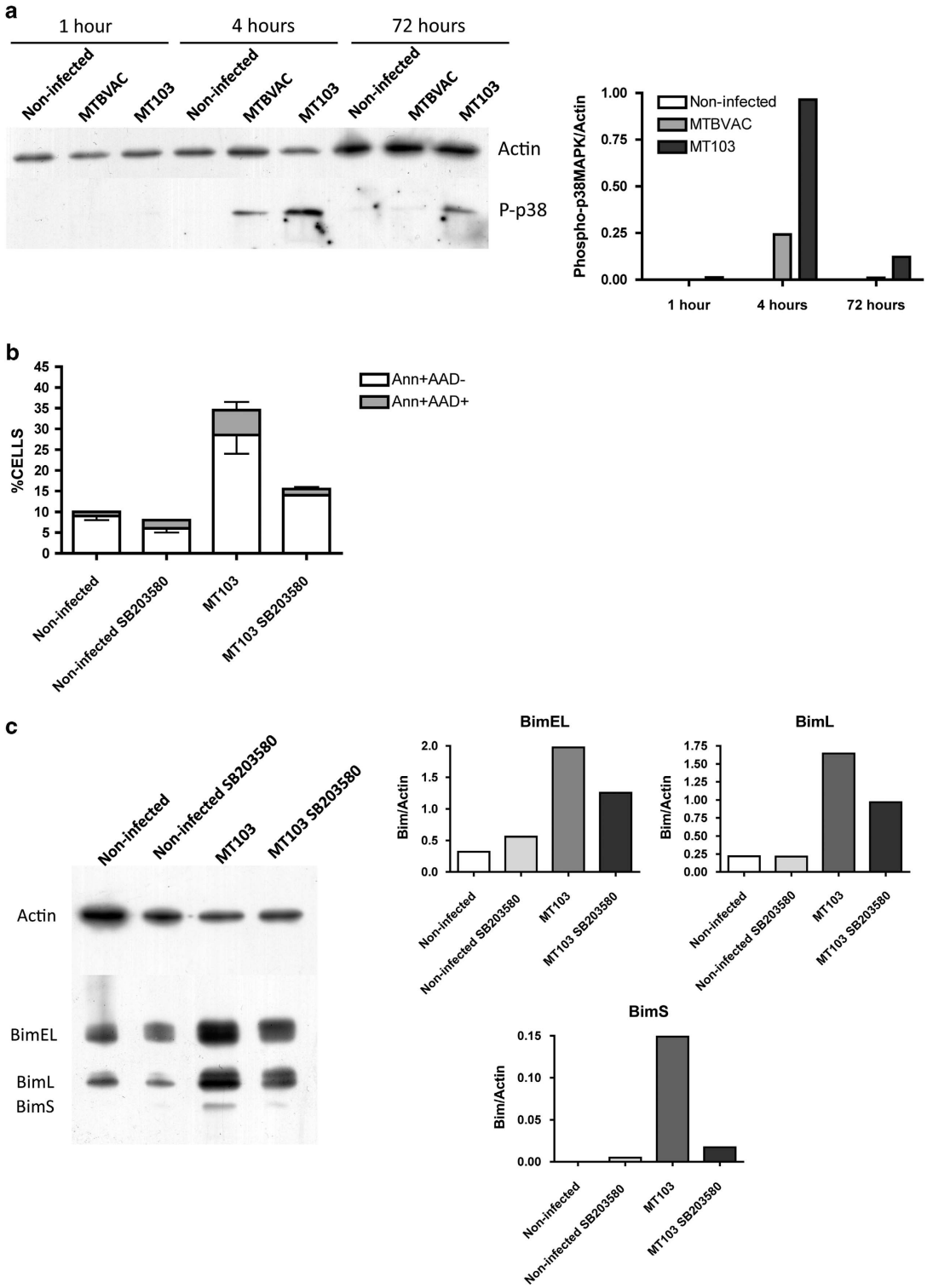

Figure 5 Bim induction triggered by MT103 is mediated by p38MAPK. (**a**) J774 cells were infected with MT103 or the live-attenuated vaccine candidate MTBVAC (MOI 10 : 1) for up to 72 h. A time course of p38MAPK phosphorylation (*P*-p38) was analysed by western blot at 1, 4 and 72 h post infection. A representative blot of two independent experiments is shown. Quantification of the western blot is represented as the ratio of phospho-p38MAPK/actin densities. (**b** and **c**) Cells were infected with MT103 for 72 h in the presence or absence of SB203580 10 μM. (**b**) Cells were stained with annexinV and 7-AAD, and analysed by flow cytometry. Data are represented as mean ± S.E.M. of two independent experiments. (**c**) A representative blot of two independent experiments is shown. Quantification of the western blot is represented as the ratio of Bim/actin densities

M. tuberculosis-induced apoptosis. Correlatively, incubation with the inhibitor reduced notably Bim expression. Data revealed that SB203580 abrogates expression of the three isoforms of Bim, even though this reduction was much more evident in the case of BimS.

Discussion

The mitochondrion is a widely used target by many pathogens for modulating cell death.[23] In the present work, we show that the pro-apoptotic member of the Bcl-2 family, the BH3-only protein Bim, is a key regulator of apoptosis induced by virulent *M. tuberculosis* clinical isolate MT103. To our knowledge, this is the first report demonstrating that a BH3-only protein is responsible for apoptosis induced by a virulent *M. tuberculosis* strain. Nevertheless, Bim has a central role in the control of host cell death by other pathogens, such as *Neisseria gonorrhoeae*, which is able to promote or inhibit apoptosis upregulating or downregulating Bim levels, respectively.[24,25] *Chlamydia trachomatis* also favours Bim degradation to inhibit host cell death.[26] Altogether, these data suggest that Bim modulation could be a general strategy exploited by different types of bacteria.

Resistance to programmed cell death by infected Bak- and Bax-deficient cells indicates pore formation in the extracellular mitochondrial membrane, which would allow release of apoptogenic factors including cytochrome *c* from the intermembrane space. Caspase-9-knockout MEF cells resistance suggests the likely formation of apoptosome structure. Apoptosis resistance of caspases 3- and 7-knockout cells indicates that, ultimately, cell death depends on activation of the executioner caspases. In the absence of Bim, the apoptotic phenotype is highly impaired, and importantly, most of the infected cells recover their clonogeneic potential indicating that these cells are resistant to any type of cell death. Translating these results to macrophages, the main target cell of *M. tuberculosis*, we demonstrate that infection with *M. tuberculosis* significantly increases Bim expression, and more importantly, downmodulation of Bim levels after transfection with specific siRNA markedly abrogates MT103-induced apoptosis. Moreover, Bim downmodulation also reduces cell-to-cell spread of *M. tuberculosis* indicating that Bim-induced apoptosis could be a principal factor that contributes to colonisation of new host cells during *M. tuberculosis* infection.

During the preparation of this manuscript, a report was published describing a role of Bim in BCG-triggered apoptosis, in an ERK1/2-dependent process.[27] Our data are discrepant with this work as we maintain that mycobacteria-induced apoptosis is a feature exclusive to virulent strains. Using our experimental settings, and in agreement with other works[8], we do not find cell death induced by BCG infection, or other attenuated strains, such as MTBVAC vaccine candidate. Nevertheless, we tested whether ERK1/2 could be involved in *M. tuberculosis*-induced apoptosis and Bim induction, and data shown in Supplementary Figure S5 clearly differ from this hypothesis. J774 incubation with MEK1 inhibitor, U0126, did not prevent MT103-induced apoptosis and importantly, Bim upregulation.

Our data indicate that virulent *M. tuberculosis* activates the canonical intrinsic apoptotic route to kill the host cell in a process that likely involves the ESX-1 secretion system, as shown by the differences found in this work between BCG and BCG:RD1 strains. However, we cannot discard the implication of other cytotoxic molecules apart from ESX-1, because MTBVAC and SO2 attenuated strains are deficient in *phoP*, a virulence factor that regulates many other mechanisms apart from the ESAT-6 secretion machinery.

The observation that the absence of Bak and Bax prevents apoptosis and cell death after *M. tuberculosis* infection is also novel. Recently, a study analysed cell death induced by *M. tuberculosis* in macrophages from Bak- and Bax-deficient mice and found that loss of mitochondrial membrane potential following infection was independent of Bak and Bax.[28] However, these experiments were performed with a high multiplicity of infection (MOI), which accounts for necrosis-like cell death,[29] making it difficult to conclude the role of Bak and Bax in *M. tuberculosis*-induced apoptosis.

Bim activity can be regulated transcriptionally by post-translational modifications, such as phosphorylation,[30] or by its interaction with other proteins, including the anti-apoptotic partner Mcl-1[31] or with DLC1 or DLC2 dynenin light chains, in the case of BimEL and BimL isoforms.[32] In this context, a previous work shows that Mcl-1 downregulation leads to an increment of H37Rv-induced apoptosis,[33] which could be explained by the release of Bim after Mcl-1 downmodulation. Our data indicate that in J774 macrophages Bim expression is upregulated at both mRNA and protein levels following infection with *M. tuberculosis*. Moreover, MT103 and MTBVAC differentially induced Bim in infected cells, with the major difference being the induction of BimS predominantly by MT103. Of note, BimS has been described as the most cytotoxic isoform of Bim,[34] with a strong capacity to directly translocate Bax to mitochondria.[35] BimS activity is mainly regulated by transcriptional activity,[36] whereas BimEL and BimL expression does not necessarily lead to apoptosis as they can be sequestered by the cytoskeleton.[32]

Our data suggest that p38MAPK has a crucial role in Bim-dependent apoptosis following MT103 infection. We detected p38MAPK phosphorylation in MT103-infected cells mainly at early time points post infection, suggesting that this is an early event necessary to trigger the subsequent apoptotic machinery. Of note, MTBVAC transiently induced p38MAPK activation at 4 h post infection, although to a lesser extent than MT103. At longer time points, only MT103 was shown to sustain p38MAPK phosphorylation. Importantly, duration of p38MAPK activation has been suggested to be crucial in the role of this kinase, promoting death or survival. Transient and rapid activation has been associated with survival and proliferation, whereas sustained activation favours cells to undergo apoptosis.[37,38]

Two main pathways have been described to control Bim expression: ER stress,[16] and the transcription factor FOXO3.[39] p38MAPK can modulate both routes. It directly phosphorylates FOXO3, promoting its translocation to the nucleus.[40] Similarly, it phosphorylates CHOP, a transcription factor induced during ER stress, enhancing its transcriptional activity.[41] It has been previously found that ESAT-6 induces different ER stress markers, including CHOP expression both

in vitro and *in vivo*.[5,13,42] Moreover, Bim has been reported to be induced in a CHOP-dependent fashion.[16] Consequently, we analysed CHOP expression in MT103- and MTBVAC-infected J774 cells and surprisingly CHOP was similarly upregulated by both strains (data not shown), indicating that CHOP induction did not correlate with cell death nor Bim increase in our model. Nevertheless, this result does not discard a role of ER stress signalling in *M. tuberculosis*-induced apoptosis, as ER stress-induced cell death can occur in a CHOP-independent way.[43,44] Remarkably, a recent paper describes a role of FOXO3 in mycobacteria-induced cell death, which could suggest that this transcription factor might be responsible for Bim induction during mycobacterial infection but more work is needed to elucidate in detail this mechanism.

Our results show that a virulent clinical isolate of *M. tuberculosis* upregulates the expression of the BH3-only protein Bim, leading to apoptosis in both fibroblast and macrophage cells through activation of the mitochondrial cell death pathway. Moreover, we show that Bim inhibition reduces cell-to-cell spread of *M. tuberculosis,* suggesting that Bim may be a critical host factor that promotes *M. tuberculosis* spread and virulence. This finding could be a potential basis for the design of new treatments targeting the molecular regulators of Bim-mediated apoptosis.

Materials and Methods

Cell culture. For this study, we used mouse embryonic fibroblasts (MEFs) immortalised with SV40 virus or using a 3T9 protocol and the mouse bone marrow-derived cell line J774 (Health Protection Agency, Cat No.85011428). All cell lines were cultured in DMEM medium supplemented with 10% inactivated fetal bovine serum (Biological industries, Reactiva, Barcelona, Spain) and 2 mM glutamine (Biological industries) at 37 °C and 5% CO_2. MEF used in this study were:

SV40-transformed WT MEF (Health Protection Agency, Cat No.98061101).

SV40-transformed MEF.Caspase 9 $-/-$ (MEF.Casp 9 $-/-$) and MEF.Caspases 3/7 $^{-/-}$ (MEF.Casp 3/7 DKO), were kindly provided by Richard A Flavell.[45]

SV40-transformed MEF.Bak $-/-$, MEF.Bax $-/-$, MEF.Bid $-/-$, MEF.Bak/Bax DKO and 3T9-transformed MEF.Bak/Bax DKO were kindly provided by Christoph Borner.

3T9 WT or MEF.Bim $-/-$ cells were kindly provided by Andreas Strasser.[46]

Bacterial strains and growth conditions. *M. bovis* BCG Pasteur 1173P2,[47] live *M. tuberculosis* attenuated vaccine MTBVAC,[7] *M. tuberculosis* clinical isolate MT103, its *phoP*-mutant derivative SO2 (kanamycin resistant) and the *phoP*-complemented SO2 strain (SO2:*phoP*)[20] were used in this study. MT103 strain was rendered fluorescent by the transfer of plasmid pMV361H *gfp* (green fluorescent protein).[48] Mycobacteria were grown at 37 °C in Middlebrook 7H9 broth (BD Biosciences, Madrid, Spain) supplemented with 0.05% Tween 80 and 10% Middlebrook albumin dextrose catalase enrichment (ADC; BD Biosciences), and when required the medium was supplemented with 20 μg/ml of kanamycin or hygromycin. The virulence and quality of our stocks are always confirmed *in vivo* in mice. All virulent stocks used to perform these experiments showed high virulence *in vivo*.

Cell culture and infections. MEF or J774 cells were cultured at 37 °C and 5% CO_2 in DMEM medium supplemented with 10% inactivated fetal 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. For some experiments, bacteria were inactivated by heat treatment for 20 min at 100 °C. Bacterial suspension for indicated MOIs was prepared in DMEM complete medium and put in contact with cells for 4 h. Afterward, cells were washed three times with PBS to remove extracellular bacteria and fresh DMEM complete medium was added. SB203580 or U0126 (Calbiochem, Madrid, Spain) inhibitors were added when corresponds at the indicated concentrations.

GeneSolution siRNA (QIAGEN, IZASA, Barcelona, Spain) was used to knockdown Bim expression in J774 cells. siRNAS were transfected with HiPerfect Transfection Reagent (QIAGEN), using the protocol supplied by the manufacturer for J774 cells transfection. siRNA1: 5′-TCCGCTTATTTAAATGTCTTA-3′; siRNA2: 5′-TGGGTAGGCCTTTGTACTTAA-3′; siRNA3: 5′-CAAGTTGTAATAAACATACAA-3′; siRNA4: 5′-CACCCTCAAATGGTTATCTTA-3′.

Apoptosis analysis *in vitro*. PS exposure and plasma membrane integrity were evaluated by AnnexinV-APC (AnnV) and 7-AAD (BD Biosciences) staining according to the manufacturer's instructions, and analysed by flow cytometry. Briefly, cells were washed and incubated with AnnV and 7-AAD in Annexin-binding buffer (ABB) for 15 min in dark at room temperature. Afterward, cells were washed with ABB and fixed with 4% paraformaldehyde (PFA) containing $CaCl_2$.

Caspase 3 activation was analysed by flow cytometry using an antibody against active form of caspase 3 (BD Biosciences), as previously described.[49]

Clonogenic assay. MEF cells were seeded in 24-well plates at approximately 5×10^3 cells per well and infected with the MT103 at MOI of 30 : 1. Seven days post infection, cells were trypsinized and 150 cells per well were seeded in a final volume of 3 ml in a 6-well plate. Cells were then allowed to grow during eight additional days at 37 °C, following which the medium was removed and cell colonies were counted following 20-min fixation with a mixture of glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) at room temperature.

Analysis of Bim expression. RNA from control and infected cells was extracted with the QIAGEN RNAeasy kit (QIAGEN), according to the manufacturer's instructions. Bim expression was analysed by RT-qPCR using the following primers: forward 5′-TGATTACCGCGAGGCTGAA-3′ and reverse 5′-ACCAGACGGAAGA TAAAGCGTAAC-3′. Primers for b-actin expression analysis were forward 5′-TGTT ACCAACTGGGACGACA-3′ and reverse 5′-CTGGGTCATCTTTTCACGGT-3′.

Cell homogenates were obtained lysing the cells with a buffer containing 0.1% Triton X-100 and protease and phosphatase inhibitors (Roche, Madrid, Spain). Protein concentration was determined by the Bradford method (BioRad, Madrid, Spain) and 10 μg of total protein was loaded in a 15% polyacrylamide gel, separated by SDS-PAGE and transferred to PVDF membrane (GE Healthcare, Madrid, Spain). Membranes were incubated with anti-β-actin (Sigma, Madrid, Spain), anti-Bim (Cell Signalling, IZASA), anti-CHOP (Cell Signalling) or anti-phospho (Thr180/Tyr182)-p38MAPK (Cell Signalling) primary antibodies according to dilutions indicated by the manufacturer. After corresponding secondary antibodies incubation, membranes were revealed using ECL plus Western Blotting system (GE Healthcare). Western blot quantification was performed by densitometry analysis using the ImageJ64 software.

Conflict of Interest

The authors declare no conflict of interest.

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