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Mycoplasma bovis delay in apoptosis of macrophages is accompanied by increased expression of anti-apoptotic genes, reduced cytochrome *C* translocation and inhibition of DNA fragmentation

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ABSTRACT

Bacterial pathogens have evolved to manipulate host cell death and survival pathways for their intracellular persistence. Understanding the ability of a bacterium to induce or inhibit cell death is essential for elucidating the disease pathogenesis and suggesting potential therapeutic options to manage the infection. In recent years, apoptosis inhibition by different bacteria has been suggested as a mechanism of survival by allowing the pathogen to replicate and disseminate in the host. *Mycoplasma bovis* has evolved mechanisms to invade and modulate apoptosis of bovine peripheral blood mononuclear cells (PBMC), red blood cells (RBCs), primary macrophages and monocytes. To date, these mechanisms are poorly understood. Using apoptosis assays such as Annexin V binding, caspases activity, reactive oxygen species production, DNA fragmentation and differential gene expression we set out to determine how *M. bovis* modulates macrophage survival. Using the BoMac cell line, we report a significant reduction in STS-induced apoptosis through caspase dependent manner. Besides activating the NF-kβ pathway and inhibiting caspases 3, 6 and 9, *M. bovis* strain Mb1 also inhibits production of reactive oxygen species and DNA fragmentation of the host cell. We also report a significant up-regulation of the anti-apoptotic genes *Bcl-2* and *Bcl-X_L* upon infection. Our results indicate that *M. bovis* strain Mb1 inhibits the intrinsic pathway of apoptosis and up-regulate survival genes in BoMac cells.

1. Introduction

Mycoplasma bovis is a bacterial pathogen that causes various diseases including chronic bronchopneumonia, mastitis, otitis, keratoconjuctivitis, meningitis, infertility and arthritis in cattle (Arcangioli et al., 2008; Caswell and Archambault, 2007; Caswell et al., 2010). The importance of *M. bovis* among other pathological agents implicated in bovine respiratory disease (BRD) complex has also been shown (Maunsell and Donovan, 2009; Maunsell et al., 2011). BRD is a multifactorial disease of feedlot cattle that is caused by *M. bovis* co-infection with bovine herpes virus (BHV-1); parainfluenza virus (PI-3); bovine respiratory syncytial virus (BRSV); bovine viral diarrhea virus (BVD) and *Mannheimia haemolytica*, *Pasteurella multocida* (Arcangioli et al., 2008). It is also the most pathogenic bovine Mycoplasma in Europe and America (Nicholas and Ayling, 2003; Snowder et al., 2006). Although *M. bovis* is considered to be an extracellular pathogen, there is *in-vivo* (Kleinschmidt et al., 2013) and *in-vitro* evidence that indicates intracellular presence and survival in different subsets of the host immune cells (Burki et al., 2015; Maeda et al., 2003; van der Merwe et al., 2010). To date, the mechanisms by which *M. bovis* interacts with host cells to generate disease are poorly understood. Naturally occurring *M. bovis* persists in a herd over an extended period and can be consistently identified not only in lesions but also commonly in healthy and pneumonic lungs (Gagea et al., 2006).

Apoptosis (Programmed cell death) is employed by host cells to maintain normal cell turnover, embryonic development and limit pathogen survival and propagation (Elmore, 2007). Apoptosis can also be exploited by the pathogen to evade the host immune response (Fulda et al., 2010; Hotchkiss et al., 2009; Renehan et al., 2001). *M. bovis* has been demonstrated to exert both pro and anti-apoptotic effects on a diversity of cell types, including neutrophils, lymphocytes, monocytes and macrophages (Jimbo et al., 2017; Mulongo et al., 2014; Suleman

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et al., 2016; van der Merwe et al., 2010). In monocytes, *M. bovis* manipulates apoptotic signalling through inhibition of caspase 9 and activation of the NF-kβ pathway (Mulongo et al., 2014). *M. bovis* strain Mb1 exerts anti-apoptotic effects on macrophage STS-induced apoptosis (Suleman et al., 2016) but the mechanisms are unknown. In this study, we used the BoMac bovine macrophage cell line to determine the apoptosis pathways modulated by Mb1. The BoMac cell line has been previously used to study *M. bovis* co-infection with bovine viral diarrhea virus in bovine macrophages (Burgi et al., 2018). In the present study, we demonstrate the ability of *M. bovis* strain Mb1 and not Mb304 to delay STS-induced apoptosis in BoMac. We also show that *M. bovis* strain Mb1 activates NF-kβ pathway and induce up-regulation of pro-survival genes Bcl-2 and Bcl-X_L; and inhibits the activity of caspases 3, 6 and 9, ROS production and DNA fragmentation upon infection. Our data continue to support observations that *M. bovis* inhibit apoptosis for survival and potentially facilitate bacterial survival, replication, and transmission.

2. Materials and methods

2.1. Bacteria strains and culture conditions

The *M. bovis* strain Mb1, isolated from the synovial fluid of a calf exhibiting signs of arthritis (Perez-Casal and Prysliak, 2007) and the *M. bovis* strain Mb304 obtained from bison lung tissue (Suleman et al., 2016) were used in all experiments. Both strains were grown in modified Hayflick's medium at 37 °C in a 5% CO_2 atmosphere. The cells were collected by centrifugation (5500 x *g* for 15 min) at the exponential phase of growth and then washed with minimum essential medium (MEM; Invitrogen, Burlington, ON, Canada). Bacteria were suspended to a cell density of 1×10^8 cfu/ml in MEM supplemented with 30% glycerol and stored at −70 °C until use.

2.2. BoMac cell line

The bovine peritoneal macrophage cell line (BoMac) was obtained as a gift from Dr. Matthias Schweizer (Universität Bern, Switzerland) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM sodium pyruvate and 10% foetal bovine serum (FBS). The cell line was grown to near confluence and split every third-day using trypsin/EDTA. The cell viability was evaluated by the trypan-blue exclusion assay as described earlier (van der Merwe et al., 2010).

2.3. Mycoplasma bovis invasion and survival in BoMac cells

The assay was carried out as previously described with a few modifications (van der Merwe et al., 2010). Briefly, 5×10^6/per well BoMac cells were infected with a range of MOIs (BoMac: *M. bovis*) of 1:0.1 and 1:5, using 5×10^6 cells/ml and 2.5×10^7 cfu/ml respectively. After 3 h incubation, the cells were washed and cultured in medium containing 400 µg/ml gentamicin (Gm, Gibco®) for 2 h to kill any extracellular bacteria. Cells were washed twice with warm D-MEM, suspended in D-MEM and incubated for 2, 6, and 18 h as shown in the scheme (Suppl. Fig. 1a). Cells were cultured in the presence or absence of Gm to ensure that the use of Gm did not interfere with the recovery of viable intracellular bacteria. The culture supernatants were collected at each time point and the adherent cells harvested and washed with PBS before lysis. The culture supernatants and lysed BoMac cells from each time point were plated in duplicates as 10-fold serial dilutions on Hayflick's agar plates and incubated at 37 °C in a 5% CO_2 atmosphere, to determine the number of extracellular and intracellular viable bacteria respectively.

2.4. Apoptosis assay

Apoptosis was detected using the Alexa Fluor®488 annexin V/Dead Cell Apoptosis kit (Molecular probes, Life technologies®, USA). Briefly, cells were infected with both strains of *M. bovis* (alive and dead) at an MOI of 1:5 (BoMac: *M. bovis*) for 24 h and incubated in D-MEM medium in the presence or absence of staurosporine (STS, 2 µg/ml for 6 h). To assess if live cells were needed to modulate apoptosis, Mb1 and Mb304 were killed with Gm (400 µg/ml) for 2 h and washed once with warm D-MEM medium prior to the apoptosis assay. STS (2 µg/ml for 6 h) was used as positive control, and the negative control included untreated cells grown in the same culture conditions. The cells were harvested using trypsin/EDTA buffer and suspended at 2×10^6 cells/ml in annexin V binding buffer. For every 100-µl aliquots of cells, 5 µl of annexin V- Alexa Fluor®488 and 1 µl of 100 µg/ml propidium iodide (PI) were added and incubated for 15 m in the dark. Finally, 400 µl of annexin V binding buffer was added to each tube, mixed gently and the cells immediately analysed by flow cytometry (BD FACSCalibur™, San Jose, CA) using Kaluza® software (Beckman Coulter, Indianapolis, US).

2.5. Caspase assay

BoMac cells were infected with both strains at an MOI of 1:5 (BoMac: *M. bovis*) for 24 h in the presence or absence of STS. Controls included cells treated with STS (2 µg/ml for 6 h) or with V-ZAD-FMK, an apoptosis inhibitor (40µM, for 24 h), after which the cells were lysed for 10 min in chilled lysis buffer. The cell debris was removed by centrifugation and the cytosolic fraction transferred into a new tube. The protein concentration was adjusted to 200 µg/ml with the cell lysis buffer provided in the colorimetric kit (Abcam, Cambridge, United Kingdom). Aliquots of 50 µl of the cleared lysate were placed into a 96-well round-bottom microtiter plate, Costar™ (Thermo Fisher scientific, Massachusetts, US) before addition of 50 µl of the 2 x reaction buffer and 5 µl of the substrates for caspases 3, 6, or 9. After incubation at 37 °C for 2 h in the dark, the samples were read in a microplate reader (xMark microplate spectrophotometer; Bio-Rad, Philadelphia, USA) at 405 nm with a 490-nm reference filter.

2.6. ROS measurement

Intracellular reactive oxygen species (ROS) was measured using the ROS-ID™ Total ROS Detection kit (Enzo Life Sciences, Inc., Plymouth Meeting, PA) following the manufacturer's instructions. BoMac cells were incubated with both strains of *M. bovis* at an MOI of 1:5 (BoMac: *M. bovis*) in the presence and absence of 50µM pyocyanin, a ROS inducer. Pyocyanin-treated cells were used as a positive control and untreated cells as a negative control. After 30 min of incubation at 37 °C with 5% CO_2, intracellular ROS production was immediately assessed by measuring intracellular green fluorescence at excitation 490 nm/emission 520 nm using flow cytometry (BD FACSCalibur™, San Jose, CA) and data analyzed with Kaluza® software (Beckman Coulter, Indianapolis, US).

2.7. Cytochrome C assay

The cytochrome *C* releasing assay was carried out following the manufacturer's instructions (Abcam, Cambridge, United Kingdom). Briefly, BoMac cells were either uninfected or infected with both strains of *M. bovis* at an MOI of 1:5 (BoMac cells: *M. bovis*) for 24 h in the presence or absence of STS (6 h) and harvested in ice-cold PBS. Cells were collected by centrifugation and the pelleted cells were suspended in buffer A on ice for 10 min. The cells were homogenized with 40 passes by using a Kontes douncer tissue grinder. The homogenates were centrifuged and the supernatants saved as the cytosol fractions. The pellets were suspended in buffer B, vortexed for 10 s and kept as the mitochondrial fractions. These two fractions were separated on 12% polyacrylamide gel and subjected to Western blotting using a bovine cytochrome *c* antibody (Abcam, Cambridge, United Kingdom) and an IRDye® 800CW goat anti-mouse IgG (LI-COR ®, Lincoln, US).

2.8. DNA fragmentation

The DNA fragmentation assay was adopted from (Dumont et al., 1999). Cells were infected with both strains of *M. bovis* for 3 h in the presence or absence of STS (2 μg/ml). Cells treated with STS alone (2 μg/ml, 6 h) were used as control. Cells were harvested using trypsin/EDTA buffer. Cells were washed twice in phosphate-buffered saline and lysed with 0.5xTBE (25 mM TRIS, 25 mM boric acid and 0.5 mM EDTA) containing 0.25% (v/v) NP-40). RNAse H (0.5 mg/ml) was added and samples incubated for 45 min at 37 °C followed by addition of Proteinase K (0.5 mg/ml, Sigma Inc, St Louis, MO, USA) with continued incubation for another 45 min at 37 °C. The cell debris was removed by centrifugation at 14 000 rpm at 4 °C for 10 min. The supernatant was transferred into a new Eppendorf tube and, the DNA fragments were separated on a 1.5% (w/v) agarose gel (50 V for 2 h), stained with ethidium bromide in TBE buffer and visualized under ultraviolet light.

2.9. NF-kβ p65 nuclear translocation assay

This assay was carried out as previously described (Mulongo et al., 2014). Briefly, both strains of *M. bovis* were used to infect BoMac at an MOI of 1:5 (BoMac: *M. bovis*) and incubated for 24 h. The controls included 1 μg/well of *Escherichia coli* LPS (Sigma Inc, St Louis, MO, USA) for 24 h, 2 μg/well of STS for 6 h and untreated cells for 24 h. The NF-kβ activation kit (Five Photon Biochemicals, San Diego, CA) was used to separate the cytoplasmic and nuclear fractions. The fractions were separated on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were performed using the bovine anti-P65 and Alexa 680 anti-rabbit antibodies provided in the kit. The only modification was that no Tween-20 was added in the blocking step as the image was taken using the LI-COR ®Odyssey® Imager.

2.10. Quantitative real-time PCR

Total RNA was extracted from BoMac cells using Trizol® (Life Technologies) from five independent experiments. Treatments included cells infected with both strains of *M. bovis*, treated with STS, and uninfected cells as control. RNA quantity was determined using a Nanodrop spectrophotometer and integrity was assessed using denaturing agarose gel electrophoresis. Reverse transcription was performed using primers in Table 1; they all had a primer efficiency of above 90%. qPCR was carried out using the RT^2 SYBR Green Fluor (Qiagen, USA) fast method performed on an iCycler i5 system. The reactions were carried out in duplicate using 5 μg of cDNA. The melting curve analysis suggested a single amplicon product. The data are expressed as fold changes as calculated using the $2^{-\Delta\Delta CT}$ method relative to the geometric mean of two stable housekeeping genes, GAPDH and β-actin.

2.11. Statistical analysis

Relative percentages of cell death, caspases activity, and differential gene expression analyses were analysed by Kruskal-Wallis tests and the multiple comparison between treated and untreated cells was done by Dunn's multiple comparisons test. The analysis of ROS was done by analysis of variance (ANOVA) and Holm-Sidak multiple comparisons test between all samples using GraphPad Prism version 7.0c for Mac OS X, GraphPad Software, La Jolla, CA, USA. Differences were considered significant it the *P* value was 0.05 or lower.

3. Results

3.1. Change Mycoplasma bovis to M. bovis

To investigate whether our *M. bovis* strains Mb1 and Mb304 can invade BoMac cells, a similar approach of viable intracellular counts analyzed using the gentamicin (GM) resistance assay was used (Burgi et al., 2018; Suleman et al., 2016). The results are shown in the supplementary Fig. 1.

After 3 h post invasion (PI), both strains were recovered in the cell lysate in similar numbers as the input cfu/ml used at the beginning of the experiment (Suppl. Figs. 1a and b). There was no growth of bacteria in the culture supernatants following Gm treatment. No differences in intracellular bacteria recovery were observed in the presence or absence of Gm in both strains (Suppl. Figs. 1a and b). There was recovery of viable intracellular bacteria from both strains 2 and 6 h after the Gm treatment. There was no recovery of the bison isolate Mb304 at 18 h of incubation (Suppl. Fig. 1b) suggesting that Mb1 is able to survive longer in BoMac cells. These findings indicated that *M. bovis* is capable of invading and persisting in BoMac cells as also shown in other cell types.

3.2. M. bovis strain Mb1 decreases STS-induced apoptosis of BoMac cells

We used Alexa Fluor®488 annexin V/PI staining to assess the modulation of apoptosis in BoMac cells by both strains of *M. bovis*. The results are shown in the supplementary Fig. 2. Cells were treated with staurosporine, a known apoptosis inducer as a positive control and others left untreated as a negative control. We also selected *M. bovis* Mb304, a bison strain, that which did not delay STS-induced apoptosis in bovine primary alveolar macrophages (Suleman et al., 2016). We included live and dead bacterial cells to address the question if the delayed apoptosis effect was only observed with live cells.

Compared to untreated cells, there was a significant increase in apoptosis after treatment with STS, STS + Mb1 and STS + Mb304 (6% vs. 73, 45, and 68% respectively). Compared to the untreated cells, there was no significant increase of apoptotic cells after treatment with dead or alive *M. bovis* Mb1 or Mb304 (Suppl. Fig. 2). Compared to STS-treated cells, there was a 38% reduction of STS-induced apoptosis (from 73% to 45%) in BoMac cells treated with live Mb1 + STS and a 43% reduction after treatment with dead Mb1 + STS (From 73% to 30%, suppl. Fig. 2). Similar to Mb1, compared to untreated cells there was a 5% decrease (from 73% to 68%) of apoptotic cells in cells treated with live *M. bovis* Mb304. Incubation with killed Mb304 resulted in a 23% reduction of apoptotic cells (From 73% to 50%, suppl. Fig. 2). Overall, these results are consistent with the delay in apoptosis by Mb1 and Mb304 previously observed in primary bovine alveolar macrophages (Suleman et al., 2016) and the delay or increase effect on apoptosis

Table 1
Primers used in this study.

Gene	Sequences (5`- 3`)	Amplicon size (bp)	Origin
BAX	F: GCTGCAGAGGATGATCGCAGCTGTG R: ATCAACTCGGGCACCTTGGTGCA	174	(Buhler et al., 2016)
Bcl-2	F: ACGGAGGCTGGGACGCCTTT R: AGGGTGATGCAAGCGCCCAC	121	(Buhler et al., 2016)
Bcl-x_L	F: CACTGTGCGTGGAAAGCGTA R: AAAGTGTCCCAGCCGCC	127	(Valdez et al., 2005)
GAPDH	F: TTCAACGGCACAGTCAAGG R: ACATACTCAGCACCAGCATCAC	119	This manuscript
β-actin	F: AGGCATCCTGACCCTCAAGTA R: GCTCGTTGTAGAAGGTGTGGT	95	This manuscript

The primer names, sequences, size of amplicons and origin are shown.

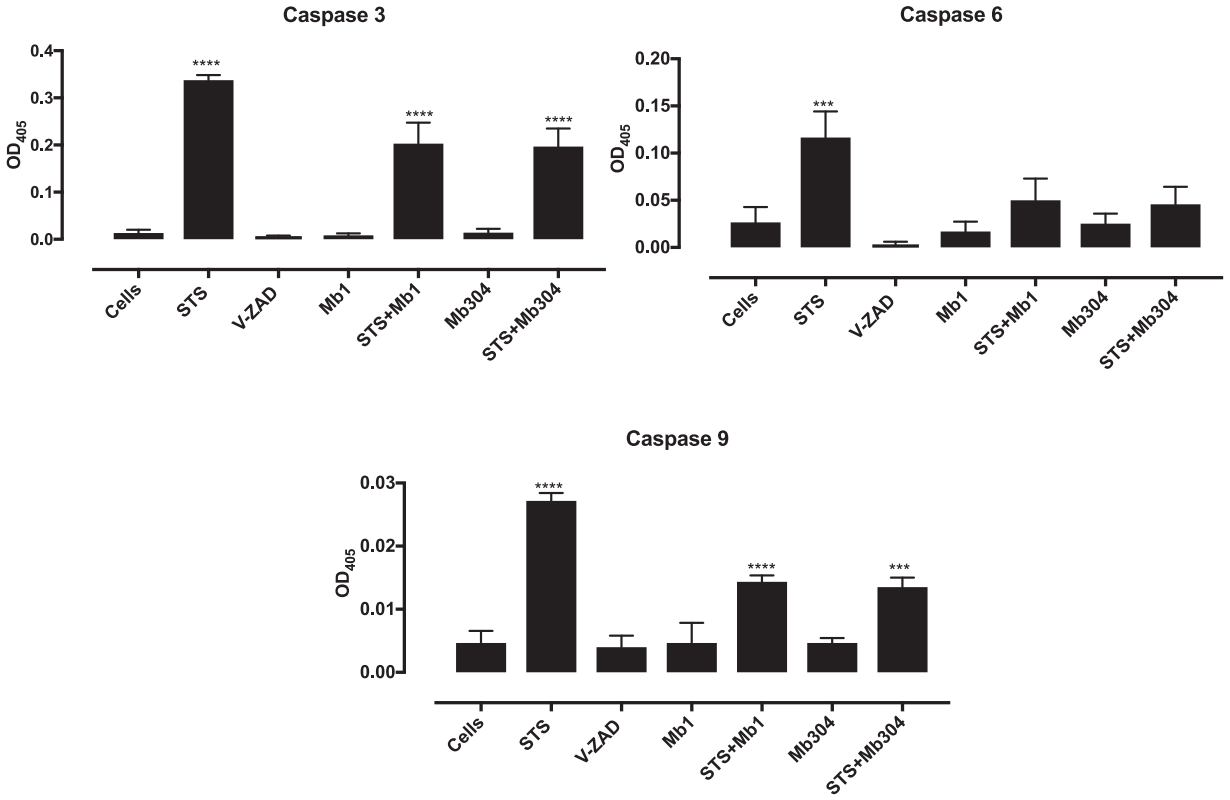

Fig. 1. Effect of *M. bovis* infection on BoMac cells caspase activity. Cells were treated with STS (6 h), Mb1 and Mb304 (3 h), in the presence or absence of STS 6 h post infection, and V-ZAD-FMK an apoptosis inhibitor (40μM, 24 h). Each column represents the average of duplicates of four independent experiments for each treatment. The bars represent the standard deviations of individual measurements. Significant differences between the treatments and untreated cells are indicated by *** = $P < 0.001$ and **** = $P < 0.0001$.

(Mb1 and Mb304 respectively) maybe found in a constitutive component as both live and dead exerted a similar effect.

3.3. *M. bovis inhibits the activities of caspases 3, 6 and 9 in BoMac cells*

Given the cardinal role of caspases in apoptosis, we investigated the modulation of BoMac STS-induced caspase activity following incubation with Mb1 and Mb304. The results are shown in Fig. 1. There was no increase in caspases 3, 6 and 9 activities after infection with both *M. bovis* strains. Incubation with the protease inhibitor Z-VAD-FMK also resulted in no increases in caspases activity (Fig. 1). Compared to untreated cells, there was a significant increase in caspases 3, 6 and 9 activities in cells treated with STS, Mb1 + STS, and Mb304 + STS (Fig. 1). Compared to cells treated with STS, there was a significant reduction of STS-induced caspases 3, 6, and 9 in cells treated with Mb1 + STS and Mb304 + STS (Fig. 1). The findings on *M. bovis* strain Mb1 are consistent with those found in bovine monocytes (Mulongo et al., 2014).

3.4. *M. bovis modulates the Reactive Oxygen species (ROS) production*

ROS is crucial in both immunological processes as a component of the killing response of immune cells to microbial invasion and an initiator for the intrinsic apoptotic-signalling cascade (Fang, 2011; Redza-Dutordoir and Averill-Bates, 2016). We investigated if *M. bovis* modulated pyocyanin-induced ROS production. The results are shown in Fig. 2. Compared to untreated cells, there was an increase in the production of ROS ($P < 0.001$) after incubation with pyocyanin, confirming that ROS is induced in these cells (Fig. 2). No changes in ROS production were detected upon infection with either of the *M. bovis* strains alone compared to untreated cells. Compared to pyocyanin-treated cells, cells treated with pyocyanin and infected with Mb1, (Pyocyanin + Mb1), showed a significant reduction ($P < 0.001$) in ROS production (Fig. 2). In contrast, compared to pyocyanin-treated cells there was no significant reduction in ROS production in cells treated with pyocyanin and infected with Mb304 (Pyocyanin + Mb304, Fig. 2). These results indicate that the cattle *M. bovis* strain Mb1 inhibited pyocyanin-induced ROS production but the bison strain Mb304 failed to decrease ROS production in the presence of pyocyanin.

3.5. *Mitochondrial cytochrome C release to the cytosol is decreased by M. bovis infection of BoMac cells*

Cytochrome *c* translocation from the space between the inner and outer mitochondrial membrane to the cytosol allows for detection of changes in the intrinsic apoptotic pathway and is required for activation of caspase 9 (Kluck et al., 1997; Wang and Youle, 2009; Wang, 2001; Yang et al., 1997). We asked whether *M. bovis* anti-apoptotic effects could prevent BoMac cytochrome *c* release to the cytosol during STS-induced apoptosis. The results are shown in Fig. 3. By comparing the mitochondrial fractions with that from cytosolic fractions we found by Western blots that there was decreased translocation of cytochrome *c* in the cytosol of uninfected and infected cells with both mycoplasma strains in the absence or presence of STS compared to the increases translocation in STS treated cells (Fig. 3a). These results were confirmed by semi quantitative band intensity analysis performed by the Odyssey® CLx Imaging System (Fig. 3b). This observation suggests that *M. bovis* decreases the release of cytochrome *c* to the cytosol a common step required for the intrinsic apoptosis pathway.

3.6. *M. bovis inhibits DNA fragmentation*

The later stage of apoptosis is characterized by DNA fragmentation (Elmore, 2007); hence, we next aimed to investigate whether the anti-

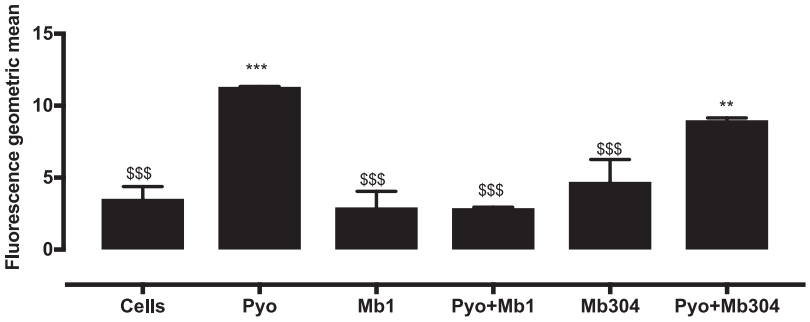

Fig. 2. Decrease in intracellular ROS with *M. bovis* infection of BoMac cells. BoMac cells were treated with both strains of *M. bovis* in the presence and absence of pyocyanin (Pyo). Pyocyanin alone and untreated cells were included as controls. FACS was used to analyse the data. Each column represents the average duplicates of two independent experiments. The number represents the fluorescence geometric mean intensity. Significant differences between the treatments and untreated cells are indicated by ** = $P < 0.01$, *** = $P < 0.001$ and significant differences between the treatments and Pyocyanin are indicated by $$$ = $P < 0.001$.

apoptotic effect of *M. bovis* on STS-induced apoptosis had any effect on decreasing DNA fragmentation. DNA integrity was assayed by agarose gel electrophoresis. The results are shown in Fig. 4. DNA fragmentation was not seen in untreated cells (lane 1) and *M. bovis*-infected cells (lanes 2 and 4). There was a slight fragmentation observed in *M. bovis* infected STS-treated cells and incubated with Mb1 or Mb304 (lanes 3 and 5). In addition, there was DNA fragmentation of the STS-treated cells as demonstrated by the tailing of the smear (lane 6) due to degradation by nucleases. These results indicate that both strains of *M. bovis* decreased DNA fragmentation in the presence of an apoptosis inducer, STS compared to STS treated cells.

3.7. NF-kβ -p65 nuclear translocation is activated in BoMac cells infected with M. bovis

As previously reported in bovine monocytes cells (Mulongo et al., 2014) incubation with *M. bovis* Mb1 resulted in the translocation into the nucleus of the p65 subunit of NF-kβ. We investigated if the effect on NF-kβ was similar in BoMac cells incubated with Mb1 or Mb304. The results are shown in the supplementary Fig. 3. Western blots show that compared to the untreated cells, there was more nuclear translocation of the p65 subunit in LPS- and Mb1- treated BoMac cells (Suppl. Fig. 3A). Similarly, there was more translocation of p65 into the nucleus in cells treated with Mb304 (Suppl. Fig. 3B). These results were confirmed by the semi quantitative band intensity analysis as determined by the Odyssey® CLx Imaging System (Suppl. Fig. 3, panels A2 and B2).

3.8. Infection of BoMac cells with M. bovis induces the expression of anti-apoptotic genes, Bcl-X_L and Bcl-2

Translocation of the NF-kβ p65 protein into the nucleus of the cell results in regulation of expression of pro- and anti-apoptotic genes (Kaltschmidt et al., 2000). We tested the effect of both *M. bovis* strains in STS-treated and untreated BoMac cells expression of the anti-apoptotic *Bcl-X_L* and *Bcl-2* genes and the pro-apoptotic *Bax* gene at 3 h and 24 h post-infection. The results are shown in Fig. 5. Relative to GAPDH and β-actin, we observed a significant increase in the level of expression

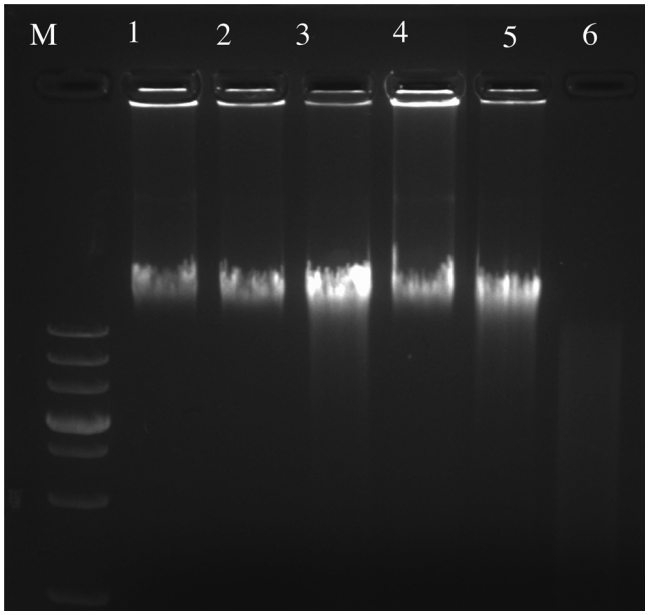

Fig. 4. DNA fragmentation analysis by agarose gel electrophoresis. DNA integrity was determined by horizontal agarose gel electrophoresis followed by staining with ethidium bromide. Lanes: M, molecular mass marker; 1: untreated cells; 2: *M. bovis* Mb1 treated cells; 3: *M. bovis* Mb1 + STS treated cells; 4: *M. bovis* Mb304 treated cells; 5: *M. bovis* Mb304 + STS treated cells and 6: STS-treated cells.

of the anti-apoptotic *Bcl-2* gene compared to the untreated cells in Mb1 (3 h)- and Mb304 (3 h)-infected cells and also an increase in expression after at a later time point (24 h) and STS + *M. bovis* although not significant compared to the control (Fig. 5). Treatment with STS alone showed no apparent differences in *Bcl-2* expression compared to the control. Similar results were observed with the *Bcl-X_L* gene. There was a significant increase of *Bcl-X_L* gene expression in Mb1 (3 h), Mb304 (3 h) and Mb304 (24 h) but also an increase in expression after STS + *M. bovis*

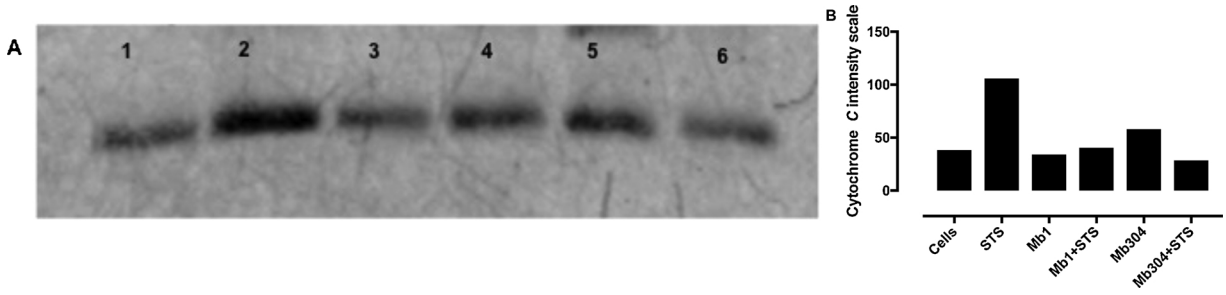

Fig. 3. Western blot analysis of *M. bovis* inhibition of mitochondrial cytochrome *c* release induced by staurosporine. **(A)** BoMac cells were treated with both strains of *M. bovis* in the presence and absence of STS as described in materials and methods. The cell samples were then fractionated and the cytosol fractions analyzed by Western blot analysis. 1: untreated cells; 2: STS-treated cells; 3: *M. bovis* Mb1 treated cells; 4: *M. bovis* Mb1 + STS treated cells; 5: *M. bovis* Mb304 treated cells and 6: *M. bovis* Mb304 + STS treated cells. **(B)** Semi quantitative intensities of the bands calculated using the Odyssey® CLx Imaging System.

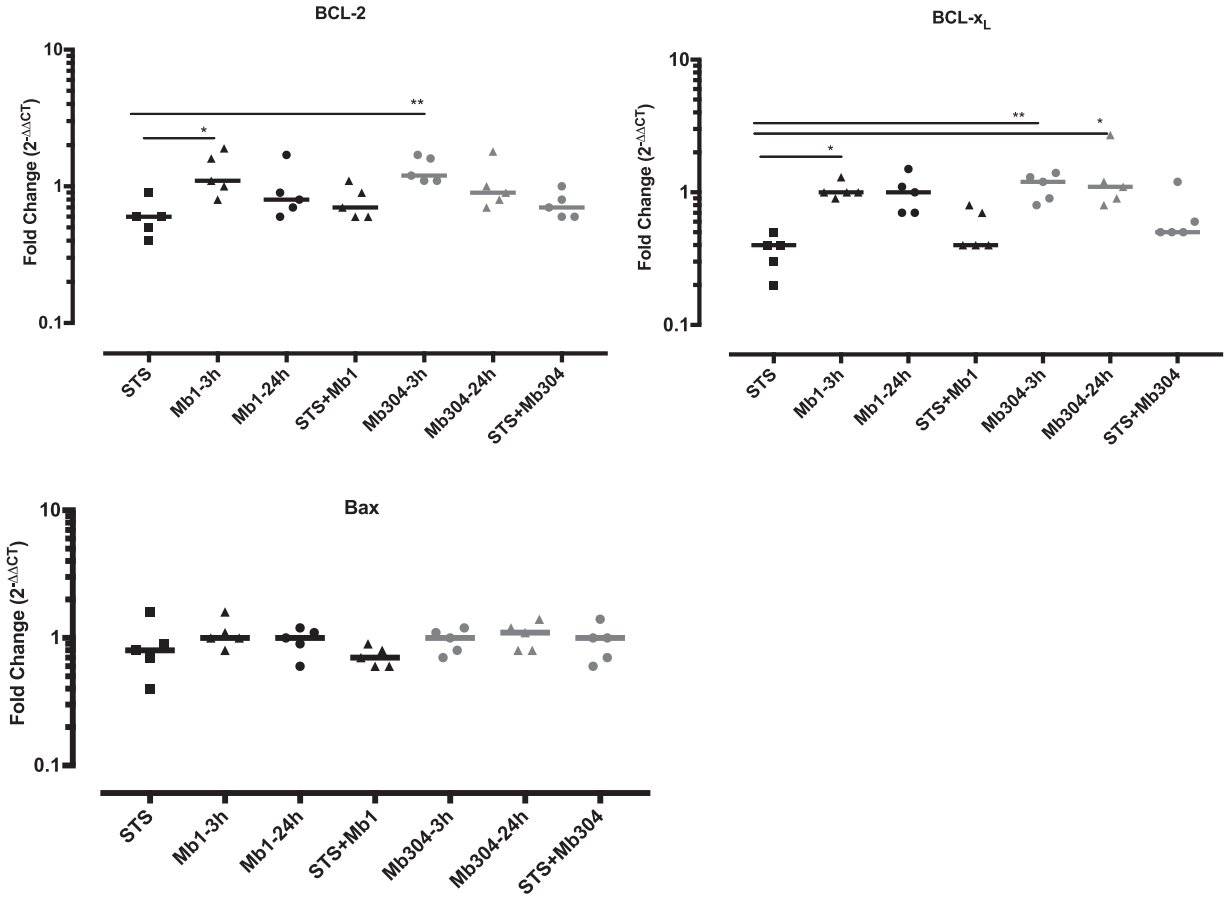

Fig. 5. *Bcl-X$_L$* and *Bcl-2* genes are upregulated in BoMac cells infected with *M. bovis* as determined by qPCR. RNA was isolated from BoMac cells infected with both strains in presence or absence of STS. Each data point shown represent the average duplicates of five independent measurements. Significant differences between the treatments and untreated cells are indicated by * = $P < 0.05$ and ** = $P < 0.01$.

although not significant (Fig. 5). However, we failed to detect any significant differences in the expression of pro-apoptotic *Bax* gene in response to infection with either strain of the *M. bovis*, STS-treated cells and un-stimulated cells (Fig. 5).

4. Discussion

Macrophages are an important line of defense against mycoplasmas in the respiratory tract (Caswell, 2014; Maunsell et al., 2011), exerting their protective effects principally through phagocytosis, production of ROS, Nitric oxide and inflammatory cytokines (Lai et al., 2010; Lohmann-Matthes et al., 1994; MacMicking et al., 1997). Mycoplasma invasion of the lungs attracts monocytes, macrophages and neutrophils; creating an inflammatory milieu that attempts to localize and eliminate the infection (Hermeyer et al., 2011, 2012). Nonetheless, there is evidence of mycoplasma persistence in both normal and diseased lungs, suggesting the existence of mechanisms by which the bacterium can evade this inflammatory assault (Khodakaram-Tafti and Lopez, 2004; Rodriguez et al., 1996).

Induction (Lancellotti et al., 2009) or inhibition (Faherty and Maurelli, 2008) of apoptosis has been reported in different pathogens and cell types and plays a vital role in disease pathogenesis (Lancellotti et al., 2009; Faherty and Maurelli, 2008). Our laboratory has previously demonstrated inhibition of apoptosis in all PBMC subsets (van der Merwe et al., 2010), monocytes (Mulongo et al., 2014) by *M. bovis* including primary bovine macrophages (Suleman et al., 2016) and the goal of the current study was to use a bovine macrophage cell line to characterize the mechanisms underlying this inhibition of apoptosis in order to identify potential pathways that could be targeted to achieve macrophage activity against mycoplasma. The bovine macrophage cell line (BoMac) is an in vitro differentiated bovine peritoneal macrophage cell line (Stabel and Stabel, 1995). It has been used to study infections with bovine herpesvirus-4 (BHV-4), *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) (Langelaar et al., 2005; Souza et al., 2007; Woo et al., 2006) and *M. bovis* co-infection with bovine viral diarrhea virus (Burgi et al., 2018). In these different studies the cell line has shown the ability to develop *bona fide* functions of alveolar macrophages including the ability to phagocytize bacteria with and without opsonization, generate reactive oxygen species (ROS), produce different cytokines (IL-6, TGFβ-1, TNFα-2, IFN-γ, IL1-α) and genes related to apoptosis (Bcl2-1) following activation and infection (Stabel and Stabel, 1995; Tooker et al., 2002). In a different study using this cell line, Abendaño showed an increased level of expression of the apoptotic inhibitor Bcl2-1 and low level of apoptosis and increased TGF-β after infection with *Map* (Abendano et al., 2013).

We first tested if our strains were capable of invading and surviving in the BoMac cell line at different multiplicity of infection (MOI) and with different infection time points using the gentamicin protection assay. These findings confirmed that there was recovery of viable intracellular Mb1 after 18 h post-invasion and Mb304 after 6 h post invasion in presence and absence of gentamicin (Suppl. Figs. 1a and b) at the different MOIs. Consistent with previous observations that gentamicin displays minimal cell membrane penetration (Elsinghorst, 1994), this antibiotic did not affect the recovery of intracellular bacteria and the viability of the cells throughout the experiment. The recovery of viable intracellular mycoplasma demonstrates the ability of both *M. bovis* strains, Mb1 and Mb304 to invade and persist in BoMac cells. Previous studies by Pilo's group also reported invasion and survival in

BoMac cells of different strains of *M. bovis,* strains JF4278 and L22/93 (Burgi et al., 2018). We further explored if both our strains (Mb1 and Mb304) were capable of delaying apoptosis in BoMac cells as previously demonstrated by our group in primary bovine alveolar macrophages (Suleman et al., 2016). Indeed, as shown previously, the cattle strain *M. bovis* Mb1 delayed STS-induced apoptosis in contrast to the bison strain, *M. bovis* Mb304 (Supplementary Fig. 2) that did not. On the contrary, the study from the co-infection experiment model of *M. bovis* with bovine viral diarrhea virus (BVD) using BoMac cells reported that the *M. bovis* strain JF4278 caused slight apoptosis and delay in STS-induced apoptosis was not observed with the type of test they used (Burgi et al., 2018). These discrepancies may also reflect the diversity that exists within *M. bovis* strains as previously described in adherence to various host cell lines that influence virulence (Thomas et al., 2003).

Successful bacteria pathogens have evolved different strategies to modulate apoptosis of various immune cells for survival and replication (Faherty and Maurelli, 2008). We further investigated which of the apoptotic pathways in macrophages was affected by infection with *M. bovis*. Different pathogenic bacteria modulate apoptosis by either protecting the mitochondria integrity (Massari et al., 2000, 2003), preventing the release of cytochrome *c* and inhibition of caspase activation (Fan et al., 1998), or activating cell survival pathways by up-regulating inhibitors of apoptosis (Abendano et al., 2013; Binnicker et al., 2003; Goebel et al., 2001; Sukumaran et al., 2004).

Caspases are cysteine proteases that are initially synthesized as inactive pro-caspases that are cleaved to an active form upon activation by apoptotic stimuli. Caspases are divided into initiators caspases (2, 8, 9 and 10) and executioner caspases (3, 6 and 7) (McIlwain et al., 2013). We observed a decrease in protease activity of caspases 3, 6 and 9 after infection with both strains of *M. bovis* compared to STS-treated cells and reduced activity in a similar trend as the caspase inhibitor (Fig. 1). In contrast, previous reports show that *M. bovis* strain Mb1 inhibits STS-induced apoptosis in monocytes by inhibiting caspase 9 but not 3. The results for caspase 6 in monocytes were inconclusive as there were no differences between untreated vs. Mb1 treated monocytes and a slight difference in Mb1 vs. STS-treated monocytes (Mulongo et al., 2014). The different observations may be attributed to the specific cell types (monocytes vs. macrophages) used for the assay. The mechanism of apoptosis of *M. bovis* has been shown to be dependent on caspase 9 an initiator caspase of the mitochondrial apoptosis pathway followed by activation of caspases 3 and 6, effector caspases that lead to DNA fragmentation and eventually cell death. Upon receiving an apoptosis signal, caspases 3 and 6 exerts their effects downstream of initiator caspase 9 (Porter and Janicke, 1999), and would be expected to be inactive upon inhibition of apoptosis.

NF-kβ is a transcription factor responsible for gene expression of target genes majority of which participate in the host immune response and cell death. Defects in NF-κβ results in increased susceptibility to apoptosis leading to increased cell death. NF-κβ regulates apoptosis by activating the expression of apoptotic or anti-apoptotic genes (Kucharczak et al., 2003). Usually, p65 is stored in an inactive form in the cytoplasm as part of the p65-p50 (NF-κβ) complex bound to the inhibitory protein IκBα. On activation, IκBα is phosphorylated by the IκB kinase leading to ubiquitination and release of the inhibitory protein from the NF-kβ complex. This, in turn, exposes nuclear import factors that target p65 to the nucleus where it binds to regulatory elements and modulates gene transcription (Rahman and McFadden, 2011). In bovine monocytes, NF-kβ activation following in vitro infection with *M. bovis* was measured by detection of accumulated p65 NF-kβ subunit in the nuclear fractions of infected cells compared to uninfected cells (Mulongo et al., 2014). Using the same assay, we observed activation of the NF-kβ signalling in BoMac cells following infection with both *M. bovis*, Mb1 and Mb304 (Suppl. Fig. 3).

Apoptosis induced by ROS production, the intrinsic pathway, leads to various events that occur in the mitochondria that include the loss of membrane potential ($\Delta\Psi_m$), cytochrome *c* release, and participation of the B-cell-lymphoma protein 2 (Bcl-2) homologs (Redza-Dutordoir and Averill-Bates, 2016; Wang and Youle, 2009; Wang, 2001). Apoptosis is regulated by two groups of modulators: the anti-apoptotic Bcl-2-family proteins (such as Bcl-X_L and Bcl-2), and pro-apoptotic family of proteins (such as Bax and Bak) (Elmore, 2007; Riedl and Shi, 2004; Shi, 2002). The pro-apoptotic proteins are mostly found in the cytosol and the anti-apoptotic proteins are localised in the outer mitochondrial membrane as heterodimers with apoptotic proteins (Bax and Bad) thus inhibiting their apoptotic functions and regulate mitochondrial $\Delta\Psi_m$, cytochrome *c* release and caspase activation (Cory and Adams, 2002). In the present study, we observed suppression of ROS production by *M. bovis* strain Mb1 as also shown in leukocytes infected with *N. gonorrhoeae* (Chen and Seifert, 2011). These findings agree with previous finding that observed no increase in neutrophil intracellular ROS levels upon stimulation with *M. bovis* (Gondaira et al., 2017; Mitiku et al., 2018). Host cells generate ROS as part of the oxidative burst during an infection to control microbial infection and its role in DNA damage during apoptosis is described in the literature (Simon et al., 2000; Wang and Youle, 2009). In addition to inhibition of ROS, we also report reduced translocation of cytochrome *c* from the mitochondrial intermembrane space to the cytosol of infected BoMac cells. Again, our results are consistent with previous reports in *N. gonorrhoeae* (Chen and Seifert, 2011), suggesting that *M. bovis* inhibits apoptosis in BoMac cells by preventing mitochondrial depolarization. The anti-apoptotic proteins, Bcl-2 family of proteins, maintain the $\Delta\Psi_m$ of the mitochondria and prevent cytochrome *c* release to the cytoplasm hence, inhibit activation of initiator caspase 9 that exerts its effects upstream of executioner caspases 3 and 6 (Sukumaran et al., 2004). We subsequently report an up-regulation of the anti-apoptotic genes, Bcl-X_L and Bcl-2 by both strains in BoMac cells. These is consistence with a study that reported up-regulation of the Bcl-2 gene in BoMac cells infected with *map* (Abendano et al., 2013). In *E. coli* infection of RAW 264.7 cell line induces the expression of Bcl-X_L (Sukumaran et al., 2004), and in *Toxoplasma gondii* infection of U937 cells induces expression of Mcl-1, another anti-apoptotic protein of the Bcl-2 family (Goebel et al., 2001) and in *Neisseria* spp., up-regulation of c-IAP-2 and Mcl-1 in primary human urethral epithelial cells was reported (Binnicker et al., 2003). Hence, we cannot exclude the involvement of additional anti-apoptotic member of the Bcl-2 protein family and IAPs.

Taken together, our findings suggest novel mechanisms that *M. bovis strain* Mb1 delays STS-induced apoptosis via the intrinsic pathway in a caspase dependent manner by inhibition of caspases 3, 6 and 9; decreased cytochrome *c* release; activation of NF-β with anti-apoptotic consequences of increased up-regulation of the anti-apoptotic survival genes *Bcl-X_L* and *Bcl-2* and absence of DNA fragmentation. In the bison strain, *M. bovis* strain Mb304 we observed similar results except for the STS-induced apoptosis assay where Mb304 induced significantly less delay in BoMac apoptosis (Fig. 2), and in primary alveolar macrophages (Suleman et al., 2016). These discrepancies between the two isolates (cattle isolate Mb1 and bison isolate Mb304) can be attributed to the difference in strain specific variations and evolutionary adaptation to the different host.

In conclusion, the ability of pathogens to inhibit apoptosis could alter the immune response and results in a successful host invasion. As a result of these studies, we have proposed a novel mechanism by which *M. bovis* prolongs the life of infected bovine alveolar macrophages by inhibiting the intrinsic apoptosis pathway. These findings present a number of potential targets for intervention along the apoptotic pathway and provide a basis for further experimentation targeting complete or partial apoptosis signalling cascades.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2018.12.004.

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