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Apoptosis of *Mycobacterium avium*-infected macrophages is mediated by both tumour necrosis factor (TNF) and Fas, and involves the activation of caspases

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SUMMARY

Mycobacterium avium causes disseminated infection in AIDS patients and several forms of infection in immunocompetent hosts. Recent studies have shown that *M. avium* infection of macrophages *in vitro* leads to apoptosis of significant numbers of infected cells. Several strains of *M. avium* used to infect human macrophages for 5 days (multiplicity of infection of 10) triggered 28–46% higher levels of apoptosis than observed with uninfected macrophages at the same time points. *Mycobacterium avium* strains unable to replicate intracellularly (rep$^-$) resulted in a 15% rate of apoptosis, while *M. smegmatis*-infected monolayers showed the same percentage of apoptotic cells as the uninfected macrophage control. The presence of anti-TNF-α antibody reduced apoptosis to 17% and the presence of anti-Fas antibody reduced apoptosis to 10%. When both antibodies were used together, the apoptosis level was 5% above the control. Treatment with TGF-β also reduced the number of apoptotic cells in infected monolayers. If intracellular growth was inhibited, apoptosis of macrophages decreased significantly. It was also shown that apoptosis was associated with IL-1β-converting enzyme (ICE) activation and was significantly reduced by a caspase inhibitor. Gaining understanding of the mechanisms of *M. avium*-associated apoptosis of macrophages will provide important insight into *M. avium* pathogenesis.

Keywords apoptosis *Mycabacterium avium* macrophages caspases

INTRODUCTION

Bacterial pathogens have developed different strategies to survive inside the host, overcome natural defences and cause disease. Killing of the immune cells might represent an advantage for the survival of pathogenic microorganisms.

A number of bacteria such as *Staphylococcus aureus, Salmonella* spp., *Shigella flexneri, Legionella pneumophila, Mycobacterium tuberculosis* as well as *M. avium* have been shown to induce apoptosis of macrophages, neutrophils and/or lymphocytes [1–6]. *Salmonella* and *Yersinia*, for example, trigger apoptosis of the host phagocytic cell before uptake occurs, suggesting that this is a mechanism to avoid phagocytosis [2,7]. Legionella and mycobacteria are intracellular organisms that induce apoptosis of host macrophages after being ingested [4–6]. *Mycobacterium tuberculosis*-associated apoptosis of macrophages and lymphocytes is observed in granulomas, and in *M. avium* infection of macrophages *in vitro* results in apoptosis [5,6,8,9].

There is debate regarding the role of host cell apoptosis in the outcome of the infection. Apoptosis of bacille Calmette–Guérin (BCG) infected macrophages induced by ATP has been shown to be associated with killing of intracellular bacilli [10]. In addition, adding exogenous hydrogen peroxide to macrophage culture also triggers apoptosis and killing of intracellular *M. avium* in macrophages [11]. In addition, Fratazzi has shown that *in vitro* apoptotic macrophages with intracellular *M. avium* can be ingested by healthy macrophages that ultimately kill the intracellular bacteria [8].

In previous studies we reported that intracellular *M. avium* when retrieved from apoptotic macrophages showed phenotypic changes and very efficiently invaded a second macrophage by alternative pathways [6]. In addition, macrophage-grown bacteria that enter an uninfected macrophage appear to live in a compartment that differs from the compartment in the initial infection [6]. Therefore, although the host may use apoptosis as a mechanism of killing *Mycobacterium*, there is evidence *in vitro* and *in vivo* that both *M. avium* and *M. tuberculosis* can exit a macrophage and infect neighbouring cells.

To gain more insight into the *M. avium*-triggered apoptosis of macrophages, we investigated the mechanisms responsible for it, with the ultimate aim of determining if the bacterium or the

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mammalian cell initiates the process and whether the intracellular bacteria can sense whether apoptosis is occurring and changes phenotypically.

MATERIALS AND METHODS

Mycobacteria

The *M. avium* strains used in this study were obtained from AIDS patients. *Mycobacterium avium* 101 (serovar 1), *M. avium* 104 (serovar 1), *M. avium* 100 (serovar 8), and *M. avium* 101 (Rep^- 170–4 obtained as reported [12]), were cultured in Middlebrook 7H10 agar for 10 days, and isolated transparent colonies were washed and resuspended in Middlebrook 7H9 broth for an additional 5 days. Prior to the assays, bacteria were washed in Hanks' balanced salt solution (HBSS) and passed through an 18 G needle 10 times. The suspension was then placed in a 15-ml polystyrene tube and vortex-agitated for 2 min. The tube was placed to rest for 5 min, after which the top 1 ml was removed and used as a source of bacteria. *Mycobacterium smegmatis* strain mC^2 155 was obtained from the laboratory of Dr W. Jacobs Jr (Albert Einstein College of Medicine, New York, NY) and was cultured as described above, with the only difference being that it was harvested after 3 days of growth. The bacterial inoculum was stained by the Ziehl–Neelson technique and observed under light microscopy to ensure complete dispersion. Only an inoculum with disperse bacteria was used in the assays. The bacterial inoculum was plated onto 7H10 agar for quantification. Bacterial viability in the inoculum was determined to be between 85% and 92% by using the LIVE-DEAD assay (Molecular Probes, Portland, OR) as previously described [6].

Monocyte-derived macrophages

Monocyte-derived macrophages were obtained from healthy donors and purified as previously described [6]. Monolayers were seeded with 5×10^5 cells. For the assays, monolayers were cultured in RPMI 1640 supplemented with 5% serum-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St Louis, MO) and 2 mM L-glutamine.

Uptake and intracellular killing assays

Monolayer infection was carried out as follows: *M. avium* or *M. smegmatis* (10^6 organisms) were added to macrophage monolayers (10^5 cells) and infection was allowed to take place for 1 h at 37°C and 5% CO_2. The monolayers were then washed three times with HBSS to remove extracellular bacteria. To lyse macrophages, the monolayers were incubated with 0·5 ml of sterile water for 10 min. Then, 0·5 ml of another lysing solution made of 1·1 ml of 7H9 medium and 0·4 ml of 0·25% SDS in phosphate buffer was added to each well for 10 more minutes. The wells were vigorously scraped with a rubber policeman and the macrophage lysates were resuspended in 0·5 ml of 20% bovine albumin in sterile water to neutralize the SDS effect. The suspension was then vortex-agitated for 2 min for complete lysis of macrophages. The macrophage lysate was briefly sonicated for 5 s (power output, 2·5 W/s) to disperse bacterial clumps and permit reproducible pour plate quantification. As a control for osmotic stability, mycobacteria were submitted to the same procedure and shown to remain 100% viable. Macrophage lysates were diluted and plated onto 7H10 agar. The plates were allowed to dry at room temperature for 15 min and incubated at 37°C for 3 days (*M. smegmatis*) or 2 weeks (*M. avium*). The results are reported as mean colony-forming units (CFU) per millilitre of macrophage lysates. Duplicate plates were prepared for each well.

Uptake of *M. avium* by macrophages was also determined by staining macrophage monolayers established in a Lab-Tek tissue culture chamber (Nunc Inc., Naperville, IL). After infection, monolayers were washed, fixed and stained with acid-fast staining. The number of intracellular bacteria was determined by counting 200 cells and the intracellular bacteria.

Reagents

Anti-human TNF-α (lot A5116071) and anti-human IL-10 antibody (lot BU176111) were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibody anti-human transforming growth factor-beta 1 (TGF-β1) was purchased from R&D Systems. A rabbit polyclonal anti-human actin was used as irrelevant control. All cytokines were purchased from Biosource Int. (Camarillo, CA). Clarithromycin was a gift from Abbott (Chicago, IL). Mouse anti-human Fas MoAb (neutralizing clone ZB4) was purchased from Medical and Biological Labs Co. (Nagoya, Japan). Caspase inhibitor Z-WEHD-FMK was purchased from Kamiya Biomedical Co. (Seattle, WA). Acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC), 7-amino-4 trifluoromethylcoumarin and 7-amino-4-methylcoumarin were obtained from Sigma. All other chemicals were obtained from Sigma.

Apoptosis assays

The presence of apoptosis in infected macrophage monolayers in comparison with uninfected monolayers was examined by Cell Death Detection ELISA (Boehringer, Mannheim, Germany) for the detection of DNA fragmentation and by the use of the TUNEL assay (Boehringer), as previously described [6].

Fluorimetric measurement of IL-1β-converting enzyme activity

IL-1β-converting enzyme (ICE) activity was measured by the liberation of 7-amino-4-methylcoumarin from Ac-YVAD-AMC at excitation and emission wave lengths of 380 and 460 nm, respectively, using the method of Thornberry [13]. Macrophage lysates were assayed at 37°C in 1·25 ml of 100 mM HEPES, 10% sucrose, 0·1% CHAPS, 10 mM DTT, pH 7·5, in a luminescence

Table 1. Apoptosis of human macrophages infected with several strains of *Mycobacterium avium*

	Percent apoptosis†	
Treatment/infection	TUNEL assay	ELISA
Control‡	3 ± 1	4 ± 2
M. avium 101	39 ± 5	42 ± 7*
M. avium 104	47 ± 3	50 ± 3*
M. avium 100	31 ± 2	30 ± 4*,**
M. avium 170–4 (Rep^-)	15 ± 2	20 ± 3*,**
M. smegmatis§	2 ± 1	2 ± 1**

† After 5 days of infection.

‡ Multiplicity of infection: 10.

§ Apoptosis was measured at day 3 for *M. smegmatis*, due to the short intracellular survival.

* $P < 0{\cdot}05$ compared with control; ** $P < 0{\cdot}05$ compared with the apoptosis induced by *M. avium* 101 and 104.

Table 2. Is bacterial replication necessary to trigger macrophage apoptosis?

Infection	Intracellular inoculum	Intracellular CFU at day 5¶	Percent apoptosis†	
			TUNEL assay	ELISA
Control§	–	–	3 ± 1	$2{\cdot}5 \pm 1$
M. avium 101	$1{\cdot}0 \pm 0{\cdot}2 \times 10^6$	$3{\cdot}5 \pm 0{\cdot}4 \times 10^7$	36 ± 5	33 ± 4*
M. avium 101 sonicate	–	–	16 ± 4	15 ± 6*,**
M. avium + clarithromycin (inhibitory)‡	$2{\cdot}1 \pm 0{\cdot}4 \times 10^6$	$2{\cdot}4 \pm 0{\cdot}2 \times 10^6$	1 ± 1	2 ± 1**
M. avium 170–4 (Rep^-)	$1{\cdot}7 \pm 0{\cdot}3 \times 10^6$	$1{\cdot}1 \pm 0{\cdot}4 \times 10^6$	10 ± 2	13 ± 4*,**

† After 5 days.
‡ Clarithromycin at 4 μg/ml was replenished daily.
§ Multiplicity of infection: 10.
¶ As described in Materials and Methods.
* $P < 0{\cdot}05$ compared with control; ** $P < 0{\cdot}05$ compared with apoptosis induced by *M. avium* 101.

fluorimeter (Cytofluor II; PE Biosystems, Framingham, MA). Assays contained 100 μg of lysate protein and 20 μm substrate. Calibration was carried out with standard solutions of 7-amino-4-trifluoromethylcoumarin and 7-amino-4-methylcoumarin.

Statistical analysis

Each experiment was performed in triplicate and repeated at least three times. The results are shown as mean ± s.d. The significance of the results between control and experimental groups was determined by Student's *t*-test.

RESULTS

Mycobacterium avium *triggers macrophage apoptosis*

Previous studies have shown that *M. avium* triggers macrophage apoptosis, but the number of strains studied has been limited. To determine if apoptosis is a common finding after macrophage infection with *M. avium*, we used three AIDS isolates, two of them very virulent in mouse models and one (*M. avium* 100) an attenuated strain in C57Bl/6 mice (data not shown). In addition, we infected macrophage monolayers with the strain 170–4, a replication minus strain, auxotrophic for methionine [12] and *M. smegmatis*. Apoptosis was measured after 5 days of infection for the *M. avium* strains and 3 days for *M. smegmatis*. As shown in Table 1, *M. avium* 101 and 104 caused the greatest level of apoptosis, whereas infection with both *M. avium* 100 and *M. avium* 170–4 resulted in significantly less macrophage apoptosis. The level of infection at time zero was similar among all strains ($3–5 \times 10^5$ organisms). Strain 100 grew more slowly than 101 and 104 ($8 \pm 0{\cdot}4 \times 10^5/10^5$ macrophages and $6 \pm 0{\cdot}6 \times 10^6/10^5$ macrophage and organisms, for 100 and 101, respectively), and 170–4 does not replicate in macrophages (but does not die either).

Based on these results, there was a suggestion that macrophage apoptosis is related to intracellular replication.

To determine whether replication was necessary in order to induce apoptosis, macrophage monolayers were infected with *M. avium* 101, *M. avium* 170–4 Rep^-, or in the presence of *M. avium* sonicate (18 min at 4°C). In addition, some *M. avium*-infected macrophage monolayers were treated with 4 μg/ml of clarithromycin, a concentration previously shown to inhibit intracellular growth. Clarithromycin treatment was initiated 1 h after bacteria were added to monolayers and continued for 5 days. As shown in Table 2, while *M. avium* 101 induced significant apoptosis of macrophages after 5 days of infection, both infection with Rep^- strain and incubation with *M. avium* sonicate resulted in significantly less apoptosis. Treatment of *M. avium*-infected macrophages with clarithromycin resulted in complete absence of apoptosis.

Table 3. Role of inflammatory and anti-inflammatory cytokines on *Mycobacterium avium*-related apoptosis of macrophages

Infection	Percent apoptosis†	
	TUNEL Assay	ELISA
Control‡	$2{\cdot}5 \pm 1$	$2{\cdot}5 \pm 1$
M. avium 101	32 ± 5	35 ± 4*
M. avium 101 + anti-IL-10§	33 ± 4	31 ± 6*,**
M. avium + IL-10 (10^2 U/ml)	34 ± 5	36 ± 3*,**
M. avium 101 + anti-TGF-β1	43 ± 3	48 ± 3*,***
M. avium 101 + rabbit irrelevant antibody	35 ± 5	34 ± 6*
M. avium 101 + TGF-β1 (10^2 U/ml)	10 ± 5	9 ± 3*,***
M. avium + IL-1 (10^2 U/ml)	30 ± 6	29 ± 5*,**
M. avium + IL-6 (10^2 U/ml)	36 ± 4	32 ± 4*,**

† After 5 days.
‡ Multiplicity of infection: 10.
§ Anti-IL-10 and anti-transforming growth factor-beta 1 (TGF-β1) (rabbit polyclonal antibody) were used in a quantity enough to neutralize 1 μg of IL-10. Antibodies were replenished daily.
* $P < 0{\cdot}05$ compared with control; ** $P > 0{\cdot}05$ compared with *M. avium* 101; *** $P < 0{\cdot}05$ compared with *M. avium* 101.

Effect of cytokines on M. avium*-related apoptosis*

Cytokines such as IL-1, IL-6, TNF-α, IL-10 and TGF-β1 are secreted from macrophages upon *M. avium* infection [14–17]. To determine whether inflammatory cytokines such as IL-1 and IL-6 or anti-inflammatory cytokines such as IL-10 and TGF-β1 have any influence on *M. avium*-related apoptosis, macrophages were infected in the presence of either recombinant cytokines or neutralizing anti-cytokine antibodies. As shown in Table 3, while neither IL-1, IL-6 or IL-10 were able to change the pattern of apoptosis, adding TGF-β1 to the infected monolayers significantly

Table 4. TNF-α and Fas trigger apoptosis in macrophages infected with *Mycobacterium avium*

Infection	Percent apoptosis†	
	TUNEL assay	ELISA
Control	2 ± 1	$3{\cdot}5 \pm 1$
M. avium 101‡	35 ± 4	42 ± 5*
M. avium 101 + anti-TNF-α antibody§	17 ± 3	21 ± 4*,**
M. avium + anti-Fas antibody§	10 ± 5	11 ± 4*,**
Anti-TNF-α antibody	2 ± 1	$2 \pm 0{\cdot}5$
Anti-Fas antibody	1 ± 1	1 ± 1
Irrelevant mouse antibody	32 ± 6	38 ± 5
M. avium + anti-Fas/anti-TNF-α	5 ± 3	7 ± 2*,**

† After 5 days of infection/treatment.

‡ Multiplicity of infection: 10.

§ Concentration necessary to neutralize 10^3 U/ml of TNF-α. Antibodies were replenished daily. Irrelevant mouse antibody (TgG_2, same isotype as anti-human TNF-α and anti-Fas antibodies) had no effect on the degree of apoptosis.

* $P < 0{\cdot}05$ compared with untreated control; ** $P < 0{\cdot}05$ compared with apoptosis induced by *M. avium* alone.

decreased the degree of apoptosis, and adding anti-TGF-β1 antibody resulted in significantly increased apoptosis when compared with the degree of apoptosis in *M. avium*-infected macrophage monolayers.

TNF-α and Fas trigger apoptosis

Given the role of both TNF-α and Fas on apoptosis [18], we examined the role of both molecules on *M. avium*-related apoptosis of macrophages. Our results indicate that neutralization of either TNF-α or Fas was associated with a significant reduction in the degree of macrophage apoptosis after 5 days. Combination of both antibodies abolished most of the apoptosis in the infected monolayers (Table 4). Previous work has demonstrated that macrophage infection with *M. avium in vitro* does not result in up- or down-regulation of the expression of TNF receptor up to 72 h of infection [19]. The effect of *M. avium* infection on the expression of Fas is currently unknown.

Role of ICE on M. avium-*related apoptosis*

Caspase (3, 8 and 9) has been implicated as part of the mechanism of apoptosis of macrophages, while ICE (Caspase 1) triggered apoptosis of macrophages by *Shigella* [20]. In order to examine if caspase activity was involved in *M. avium*-related apoptosis, we used an enzymatic assay that allows quantification of ICE activity over time. The results showed that ICE activity is greater from days 3–7 after infection when large degrees of apoptosis are detected in infected monolayers compared with days 1 and 2 (Table 5).

To confirm that caspases are involved in *M. avium*-triggered apoptosis, macrophages were infected and in some monolayers Z-WEDH-FMK (5–20 μm), an inhibitor of caspases 1, 4 and 5, was added. Z-WEDH-FMK has no toxic effect on the monolayer (data not shown). The degree of apoptosis was then measured at days 2, 3, 5 and 7 by ELISA. As shown in Table 6, the use of Z-WEDH-FMK inhibited apoptosis in a dose-related manner, up to approx. 80% of apoptosis at days 2, 3, and 5, and 65% of apoptosis at day 7 at a dose of 20 μm.

DISCUSSION

In this study we demonstrated that infection of macrophages with a number of AIDS-derived *M. avium* strains triggers apoptosis, although the level of apoptosis is significantly reduced in macrophages infected with a Rep^- strain of *M. avium*. Our results also suggest that apoptosis is triggered more efficiently by replicating bacteria and is suppressed by clarithromycin, an anti-microbial with inhibitory activity against *M. avium*. Apoptosis has been suggested as a mechanism used by the host to kill intracellular mycobacteria or inhibit its growth. BCG-infected macrophages have been shown to undergo apoptosis following treatment of the monolayers with ATP [10,21], resulting in the killing of the intracellular bacteria. Likewise, it has been shown that exposure of *M. avium*-infected macrophages to hydrogen peroxide, but not to anti-Fas antibody, is associated with apoptosis and inhibition of the growth of intracellular organisms [11]. More recently, Fratazzi and colleagues [8] reported 30% apoptosis in monolayers infected with *M. avium* with a 90% reduction in the viability of intracellular bacteria. Our earlier findings agree with observations that intracellular *M. avium* induces apoptosis of macrophages, without the need for any extracellular products. However, in an infected monolayer intracellular *M. avium* grows over time and if one retrieves only bacteria from apoptotic macrophages, these intracellular bacteria can be shown to possess an invasive phenotype that differs from the phenotype expressed by bacteria cultured on plates [6]. These results are supported by the observation *in vivo* indicating that the host is not infected with thousands of bacilli, but

Table 5. Activity of IL-1β-converting enzyme (ICE) (Caspase 1) in macrophages infected with *Mycobacterium avium* over time

Experimental groups	Days after infection	ICE activity (pmol/mg)*
Control	7	Undetectable
M. avium + macrophages†	2	Undetectable
M. avium + macrophages	3	514 ± 30
M. avium + macrophages	5	637 ± 45
M. avium + macrophages	7	325 ± 36

* ICE activity was measured as the release of 7-amino-4-methylcoumarin as described in Material and Methods.

† Multiplicity of infection: 10.

Table 6. Effect of Z-WEHD-FMK (caspase inhibitor) on *Mycobacvterium avium*-triggered apoptosis of macrophages

Macrophages	Days	Percent of apoptosis			
		2	3	5	7
Uninfected		0·5 ± 0·1	2·1 ± 0·7	3·9 ± 1·1	6·4 ± 0·9
M. avium-infected†		7 ± 3	28 ± 4	45 ± 6	57 ± 9
M. avium + Z-WEHD-FMK (5 μM)‡		5 ± 2	18 ± 5	29 ± 8	35 ± 6
M. avium + Z-WEHD-FMK (10 μM)		5 ± 3	12 ± 2	17 ± 4	23 ± 5
M. avium-infected + Z-WEHD-FMK (20 μM)‡		3 ± 0·4	4·4 ± 1·5*	9 ± 1·7*	16 ± 3*
Z-WEHD-FMK (20 μM) control		0·6 ± 0·2	0·9 ± 0·3	3·7 ± 1·8	7·9 ± 2·1

† Multiplicity of infection: 10.
‡ Vehicle (diluent) had no inhibitory effect on apoptosis.
* $P < 0·05$ compared with *M. avium*-infected macrophages.

rather dozens or hundreds of bacteria that infect cells, replicate and subsequently leave those cells to infect new cells. Therefore, it is plausible to hypothesize that invasive phenotypes represent the state of readiness of the bacteria to invade neighbouring cells and escape killing mechanisms. In fact, a similar observation was recently reported by McDonough & Kress with *M. tuberculosis* [22].

An important aspect of *M. avium*-related apoptosis is the possible need for replication to induce apoptosis. Our findings showed that a Rep^- strain which is methionine auxotrophic (Bermudez *et al*., in preparation) and does not replicate intracellularly did induce apoptosis (although at a significantly lower level than that triggered by the wild type). In support of our observation, a recent study has shown that extracts of *M. avium* can induce apoptosis of human macrophages [9], suggesting that replication is not necessary. It is possible that *M. avium* products, when added to monolayers, trigger the release of cytokines that subsequently induce macrophage apoptosis, not necessarily using the same pathway(s) used by intracellular bacteria. Our results may also be explained by the well documented anti-inflammatory properties of clarithromycin [23], and potentially inhibitory effect on apoptosis.

The mechanism of apoptosis by *M. avium*-infected macrophages has been shown to be dependent on TNF-α [8]. Our results confirm the previous finding and add that Fas is also important to the process. The fact that two initial pathways induce apoptosis of macrophages infected with *M. avium* suggests the possibility that two triggering mechanisms are happening simultaneously. Nonetheless, both receptor pathways, TNF-R1 and Fas, share many similarities, including a common reliance on protein-containing 'death domains.' Whether both mechanisms are linked, or are triggered by the bacterium, is currently unknown.

Use of two different methods suggests that caspases are involved with the mechanism of apoptosis of *M. avium*-induced macrophages. The family of cysteine-proteases, identified by their homology with the nematode death gene *ced*-3, are executors of the apoptotic program in vertebrate cells [24,25]. Two members of the TNF receptor family, CD95 (Fas/APO-1) and TNFR1 (p55), are known to signal apoptosis [18]. The cytoplasmic domains of CD95 and TNFR1 share a related 'death domain' that activates an ICE protease cascade [18,25]. Another pathogen, *Sh. flexneri*, has been shown to induce apoptosis of macrophages by ICE-mediated mechanisms, and a *Shigella* product, *IpaB*, is sufficient to trigger apoptosis [26]. Whether *M. avium*-related apoptosis is triggered by a bacterial product is currently unknown. Recent work has shown that ATP can trigger apoptosis of BCG-infected macrophages, and that ICE activation is not associated with ATP-induced apoptosis [10,21]. ATP is found inside cells in a concentration between 5 and 10 nmol. Hence, cell destruction may release ATP. However, there is no current evidence for ATP-mediated apoptosis of *M. avium*-infected macrophages. Recently, *M. bovis* BCG was shown to down-regulate the expression of Bcl-2 protein in infected macrophages, which is a new mechanism by which mycobacterial infection results in apoptosis of macrophages [27].

In conclusion, we have extended the current knowledge on macrophage apoptosis following *M. avium* infection. Elucidation of several hypotheses associated with this phenomenon will require *in vivo* studies.

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