


IMMUNE CHECKPOINTS

Checkmate on cancer.

In vivo validated mouse anti-mouse immune checkpoint (IC) mAbs
Biosimilar IC mAbs with various IgG isotypes
Functionally validated IC-expressing cells



The Journal of Immunology

RESEARCH ARTICLE | JANUARY 01 2003

Survival of *Mycobacterium tuberculosis* in Host Macrophages Involves Resistance to Apoptosis Dependent upon Induction of Antiapoptotic Bcl-2 Family Member Mcl-1¹ **FREE**

Laura M. Sly; ... et. al

J Immunol (2003) 170 (1): 430–437.

<https://doi.org/10.4049/jimmunol.170.1.430>

Related Content

Translationally Controlled Tumor Protein–Mediated Stabilization of Host Antiapoptotic Protein MCL-1 Is Critical for Establishment of Infection by Intramacrophage Parasite *Leishmania donovani*

J Immunol (June,2022)

Mycobacterium tuberculosis Promotes Apoptosis in Human Neutrophils by Activating Caspase-3 and Altering Expression of Bax/Bcl-x_L Via an Oxygen-Dependent Pathway

J Immunol (June,2002)

Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors.

J Immunol (April,1993)

Survival of *Mycobacterium tuberculosis* in Host Macrophages Involves Resistance to Apoptosis Dependent upon Induction of Antiapoptotic Bcl-2 Family Member Mcl-1¹

Laura M. Sly,^{2*‡} Suzanne M. Hingle-Wilson,^{2*†} Neil E. Reiner,^{*‡} and W. Robert McMaster^{3*†}

Mcl-1 protein expression was found to be up-regulated during infection with virulent *Mycobacterium tuberculosis* strain H37Rv. Mcl-1 induction in THP-1 cells was optimal at a multiplicity of infection of 0.8–1.2 bacilli per macrophage and was independent of opsonin coating of the bacteria. Mcl-1 expression was elevated as early as 4 h, peaked at 5.8-fold above control cells at 24 h, and remained elevated at 48 h after infection. In THP-1 cells, *mMcl-1* mRNA was induced by infection with live H37Rv but not with attenuated *M. tuberculosis* strain H37Ra, heat-killed H37Rv, or latex beads. In THP-1 cells and monocyte-derived macrophages (MDMs), Mcl-1 protein was induced by infection with live H37Rv but not with attenuated *M. tuberculosis* strain H37Ra, heat-killed H37Rv, or latex beads. Treatment of uninfected, H37Ra-infected, and H37Rv-infected THP-1 cells and MDMs with antisense oligonucleotides to *mcl-1* reduced Mcl-1 expression by >84%. This resulted in an increase in apoptosis of both MDMs and THP-1 cells that were infected with H37Rv, but not cells that were uninfected or infected with H37Ra. Increased apoptosis correlated with a decrease in *M. tuberculosis* CFUs recovered from antisense-treated, H37Rv-infected cells at 4 and 7 days after infection. In contrast, CFU recoveries from sense-treated, H37Rv-infected cells or from antisense- or sense-treated, H37Ra-infected cells were unchanged from controls. Thus, the antiapoptotic effect of the induction of Mcl-1 expression in H37Rv-infected macrophages promotes the survival of virulent *M. tuberculosis*. *The Journal of Immunology*, 2003, 170: 430–437.

Mycobacterium tuberculosis, the causative agent of pulmonary tuberculosis, infects one-third of the world's population (1). It accounts for more deaths each year than any other single infectious bacteria (2). Its ability to survive and replicate in the host macrophage is critical to its pathogenesis, emphasizing a need for a clearer understanding of its interactions with the host macrophage. In vitro experimental models have demonstrated that *M. tuberculosis* infection causes apoptosis of host macrophages. This has been demonstrated by the detection of annexin V binding to surface-exposed phosphatidylserine early after infection with *M. tuberculosis* (3). In vitro infection of human macrophages with *M. tuberculosis* strain H37Ra or strain H37Rv caused TNF- α -dependent apoptosis demonstrated by a genomic DNA ladder, nuclear fragmentation, and condensation as well as by TUNEL labeling (4). Keane et al. (5) demonstrated further that avirulent *M. tuberculosis* strains and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)⁴ cause more apoptosis than virulent

strains, implicating a role for apoptosis in host defense against *M. tuberculosis*.

In addition, there is evidence that apoptosis occurs in vivo during the course of pulmonary tuberculosis. Placido et al. (6) reported that bronchoalveolar lavage from patients with reactive or disseminated tuberculosis demonstrated a 3-fold increase in apoptotic cells compared with control patients as determined by propidium iodide staining, TUNEL labeling, and tissue transglutaminase expression. Furthermore, morphological changes consistent with apoptosis have been noted in epithelioid cells from granuloma of tuberculous patients (7).

Apoptosis during *M. tuberculosis* infection has been shown to be an innate mechanism of host defense. Apoptosis, but not necrosis, was shown to reduce *M. tuberculosis* CFUs recovered during in vitro infection of monocyte-derived macrophages (MDMs) (8). Induction of apoptosis in *Mycobacterium avium*-infected macrophages has been demonstrated to be the cause of intracellular growth restriction induced by picolinic acid (9). Fas ligand (FasL), TNF- α , and ATP-induced apoptosis all caused a reduction in *M. tuberculosis* strains H37Ra and H37Rv recovered from infected macrophages in vitro (8, 10).

However, it has been demonstrated that Fas-FasL interactions and ATP-induced apoptosis are not responsible for the intracellular death of *M. tuberculosis*. CD4⁻CD8⁻ cytotoxic T cells mediated Fas-FasL interactions but did not cause the death of intracellular mycobacteria (11). Rather CD8⁺ T cells can cause the death of intracellular *M. tuberculosis* and other pathogens by a direct granule-dependent mechanism involving the release of the effector protein granzyme (12). The effect of ATP-induced killing of the intracellular *M. tuberculosis* is not due to apoptosis but rather has been attributed to enhanced phagosome-lysosome fusion and acidification of the *Mycobacterium*-containing phagosome (13–15).

*Immunity and Infection Research Center, Vancouver Hospital and Health Sciences Center, Departments of [†]Medical Genetics and [‡]Medicine (Division of Infectious Diseases), Faculty of Medicine, and [§]Department of Microbiology and Immunology, Faculty of Science, University of British Columbia, Vancouver, British Columbia, Canada

Received for publication December 12, 2001. Accepted for publication October 25, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was supported by Canadian Institutes for Health Research Grants MOP-7399 (to W.R.M.) and MOP-8633 (to N.E.R.).

² L.M.S., and S.M.H.-W. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. W. Robert McMaster, Jack Bell Research Center, 2660 Oak Street, Vancouver, British Columbia, Canada V6H 3Z6; E-mail address: robm@interchange.ubc.ca

⁴ Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; MDM, monocyte-derived macrophage; FasL, Fas ligand; MOI, multiplicity of infection; HK, heat-killed

M. tuberculosis strain H37Rv; LB, latex beads; s-oligo, phosphorothioate-modified oligonucleotide; Q-PCR, quantitative PCR; sTNFR2, soluble TNFR 2.

Proapoptotic and antiapoptotic members of the Bcl-2 family have been shown to be differentially regulated by mycobacterial infection. Bcl-2 family members are homologues of the prototypical antiapoptotic protein Bcl-2 (16). The antiapoptotic family member *bfl-1*, which encodes the protein A1, is up-regulated in macrophages in response to infection with *M. bovis* BCG (17). The antiapoptotic gene *Bcl-x_L* has also been shown to be up-regulated during *M. tuberculosis* infection with a coordinate down-regulation of Bcl-2 (18). In neutrophils, *M. tuberculosis* infection results in an increase in proapoptotic family member Bax and down-regulation of antiapoptotic family member Bcl-x_L, and these events were shown to be dependent on the generation of reactive oxygen species (19). In an animal model of primary murine tuberculosis, Bcl-2 was up-regulated while Bax was down-regulated, resulting in a net decrease in apoptosis in the host (20).

Mcl-1 is an antiapoptotic member of the Bcl-2 family. Prosurvival members of the Bcl-2 family may prevent apoptosis by maintaining the integrity of the mitochondrial membrane, thereby preventing release of cytochrome *c*, activation of caspases, and DNA degradative enzymes (21, 22). The Bcl-2 homologue, Mcl-1, is present in cells of the hemopoietic lineage (14–16, 23–25). In polymorphonuclear cells, antisense oligonucleotides to *mcl-1* have been used to reduce Mcl-1 protein expression, resulting in increased apoptosis in response to aging and hypoxia (26).

To study the role of Mcl-1 in *M. tuberculosis*-infected macrophages, we examined steady-state levels of *mcl-1* mRNA and Mcl-1 protein expression in *M. tuberculosis*-infected THP-1 cells and the association of Mcl-1 levels with bacterial survival.

Materials and Methods

Reagents and chemicals

RPMI 1640 and HBSS were obtained from StemCell Technologies (Vancouver, BC, Canada). FCS was purchased from HyClone Laboratories (Logan, UT). Middlebrook 7H9, 7H10, and Middlebrook OADC enrichment were obtained from Difco (Detroit, MI). The Kinyoun staining kit was purchased from BBL Microbiology Systems (Cockeysville, MD). Rabbit polyclonal anti-human Mcl-1 Ab was obtained from Upstate Biotechnology (Lake Placid, NY). Goat polyclonal anti-actin Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary goat anti-rabbit Ab conjugated to HRP was obtained from Bio-Rad (Hercules, CA) and secondary anti-goat Ab conjugated to HRP was purchased from Santa Cruz Biotechnology. TRIzol and Superscript Reverse Transcriptase II were obtained from Life Technologies (Grand Island, NY). The Primer Express program and SYBR green master mix were purchased from Applied Biosystems (Foster City, CA). The pGEMT-Easy vector was obtained from Promega (Madison, WI). Annexin V-PE was purchased from BD Pharmingen (San Diego, CA). Actinomycin D was obtained from Calbiochem (La Jolla, CA). LipofectAMINE was purchased from Life Technologies. Phosphorothioate-modified oligonucleotides (s-oligos) were synthesized and purified by Life Technologies.

Isolation and culture of human MDMs

MDMs were isolated as described previously (27). Mononuclear cells were allowed to adhere to cell culture flasks for 45 min at 37°C, 5% CO₂, in a humidified atmosphere. Nonadherent cells were removed with three washes with HBSS and medium was replenished. Adherent cells were used the following day for infections.

M. tuberculosis infections

The human promonocytic cell line THP-1 was obtained from the American Type Culture Collection (TIB-202; Manassas, VA). *M. tuberculosis* strains H37Rv and H37Ra were used as virulent and attenuated type strains, respectively. The human myeloid cell line THP-1 was used as a model for human macrophage cells in infection studies because of its similarity to human MDMs and its availability for use (28, 29). THP-1 cells were cultured in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine and were maintained between 2 and 10 × 10⁵ cells/ml. *M. tuberculosis* was grown to late log phase in Middlebrook 7H9 with OADC. Aliquots were frozen at -70°C. Representative samples were thawed and CFUs per ml were enumerated by plating on Middlebrook 7H10 with OADC. Heat-

killed bacteria were prepared by heating aliquots at 80°C for 2 h, resulting in a decrease in CFUs recovered of greater than five logs. Cells were infected as described previously (29). Briefly, THP-1 cells were seeded in six-well flat-bottom tissue culture plates and allowed to adhere and differentiate at 37°C in a humidified, 5% CO₂ atmosphere for 18 h in the presence of 20 ng/ml PMA. Cells were washed and medium was replaced to remove PMA 4 h before the addition of bacteria. Where indicated, bacteria were opsonin coated by incubating with 50% fresh serum in RPMI 1640 for 30 min before addition to culture. Bacteria, opsonin coated or not, were added to THP-1 cells at infection ratios of 5:1, 20:1, 50:1, or 200:1, resulting in infection rates as reported in *Results*. Latex beads (LB) were used at a ratio of 50:1, resulting in a rate of one to seven beads per cell in ~90% of cells. Nonopsonin-coated bacteria were added to MDMs at a ratio of 5:1, resulting in an infection rate of one bacilli per macrophage. Infection was evaluated by CFU assays. After 4 h of coinoculation of bacteria or LB with cells, cells were washed three times with HBSS to remove noningested particles and the medium was replenished.

SDS-PAGE and Western immunoblotting

SDS-PAGE was performed according to the method of Laemmli (30). Western-blotted membranes were developed by ECL as previously described (31).

Quantitative PCR (Q-PCR)

Total RNA was isolated from THP-1 cells using TRIzol according to the manufacturer's instructions. cDNA was prepared by reverse transcription using Superscript Reverse Transcriptase II according to the manufacturer's instructions. Q-PCR primers for *mcl-1* were designed using the Primer Express program and sequences were as follows: forward, 5'-TGGGTT TGTGGAGATTCTTCCA-3'; and reverse, 5'-ACACCTGCAAAAGCCA GCA-3'. Primers were synthesized by Applied Biosystems. The *mcl-1* Q-PCR product was cloned into pGEMT-Easy vector according to the manufacturer's instructions to compare unknown samples to those with known copy numbers of the *mcl-1* PCR product. Q-PCR was conducted on cDNA samples and dilutions of plasmid containing the *mcl-1* PCR product in triplicate using the SYBR green master mix. Negative controls included RNA with no reverse transcriptase for DNA contamination and no template for environmental contamination.

Sense and antisense s-oligos

s-Oligos were prepared to the sense and antisense strands of *mcl-1* and incorporated into cells as described previously (29). Briefly, s-oligos to human *mcl-1* were synthesized and HPLC purified by Life Technologies. s-Oligos were phosphorothioate-modified to prevent intracellular degradation and purified to remove incomplete synthesis products. The 19-mer antisense sequence was 5'-GGGGCTTCATCTCCTCAA-3' as described previously (26) and the sense sequence was 5'-TTGAGGAGATGGA AGCCCC-3'. To incorporate s-oligos into THP-1 cells, 5 × 10⁶ were resuspended in 500 μl of RPMI 1640 containing 2.5% LipofectAMINE/5 μM s-oligos and incubated on a rotary shaker for 4 h at 37°C before differentiation with PMA for THP-1 cells or before allowing monocytes to adhere for MDMs.

Annexin V-PE binding

Cells were scraped, spun down, and washed twice with 1 ml of cold PBS/10⁶ cells. Cells (1 × 10⁶) were resuspended in annexin V-PE binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂) at 10⁶ cells/ml. An aliquot (100 μl) was removed and 5 μl of annexin V-PE was added. The mixture was vortexed gently and incubated for 15 min at room temperature in the dark. The cells were washed once with binding buffer and fixed in 30% formaldehyde in methanol. The volume was increased to 500 μl with binding buffer for analysis by flow cytometry.

TUNEL assays

TUNEL assays were performed using the Fluorescein-FragEL DNA fragmentation kit (Oncogene Research Products, San Diego, CA) according to the manufacturer's instructions. Treated cells were cytospun onto slides, stained, and examined by fluorescent microscopy. Cells were considered apoptotic if they were TUNEL positive (green fluorescent nuclear staining). A positive control included incubation of THP-1 cells with actinomycin D (50 ng/ml for 19 h).

Determination of CFUs

Enumeration of CFUs was performed as described previously (29, 32). Bacilli were plated immediately after 4 h of coinoculation with cells and

washing (time 0) and at 4 and 7 days after infection. Bacilli were released from cells in cold PBS/0.1% Triton X-100, serially diluted in Middlebrook 7H9 with OADC, and 20 μ l of three dilutions were plated on Middlebrook 7H10 with OADC in triplicate. CFUs were counted after 14 days of incubation at 37°C and plates were maintained for 21 days to ensure that no additional CFUs appeared.

Statistical analyses

Data presented are expressed as means \pm SD. Statistical analyses for Q-PCR, annexin V-PE binding, and TUNEL assays were performed by an unpaired Student's *t* test. Comparisons for CFUs were done by ANOVA for each time point. Differences were considered significant at a level of *p* < 0.05.

Results

Mcl-1 is up-regulated in *M. tuberculosis*-infected THP-1 cells

Mcl-1 protein levels were determined at several *M. tuberculosis* infection ratios and at different times after infection by Western blotting. Multiplicity of infections (MOIs) for nonopsonized and opsonized *M. tuberculosis* strain H37Rv were titrated to determine the optimal MOI for inducing Mcl-1. Infection with nonopsonized bacteria at initial ratios of 5:1, 20:1, 50:1, or 200:1 resulted in MOIs of 0.04, 0.44, 0.81, or 1.80 bacteria per macrophage, respectively, as determined by recovery of CFUs immediately after infection and washing (time 0). Infection at the same ratios with opsonized bacteria resulted in MOIs of 0.59, 1.20, 4.21, and 8.56, respectively. Control cells constitutively expressed low levels of Mcl-1 and enhanced expression of Mcl-1 was observed in macrophages infected with either nonopsonized or opsonized *M. tuberculosis* (Fig. 1A). With nonopsonin-coated bacteria, induction of Mcl-1 protein occurred with a MOI of as low as 0.44 and was maximal at 0.81 (Fig. 1A, lanes 2–5). With opsonized bacteria, induction of Mcl-1 was detected at a MOI of 0.59 and was maximal at 1.20 (Fig. 1A, lanes 6–9).

Mcl-1 induction by H37Rv infection occurred early after infection. Nonopsonin-coated H37Rv was added to differentiated THP-1 cells at a 50:1 ratio and THP-1 cell lysates were prepared at different times after infection to determine the optimal time for Mcl-1 induction. The amount of Mcl-1 protein present in THP-1 cells was increased as early as 4 h after infection with a maximum at 24 h and that was maintained through 48 h after infection (Fig. 1B).

Induction of *mcl-1* mRNA levels and protein expression requires infection with virulent strain H37Rv

Expression of *mcl-1* mRNA was evaluated in THP-1 cells that were infected with either H37Rv or H37Ra as well as in cells exposed to either HK H37Rv or LB. In comparison to control cells, there was a 9.6-fold increase in *mcl-1* mRNA in H37Rv-infected macrophages (Fig. 2A). In contrast, no significant increases in *mcl-1* gene expression were observed for cells exposed to any H37Ra bacteria, heat-killed *M. tuberculosis* strain H37Rv (HK) bacteria, or LB (Fig. 2A). In THP-1 cells, densitometry of ECL-developed bands revealed a 4.8-fold increase of Mcl-1 protein, as determined by Western blotting of whole cell lysates, in H37Rv-infected cells vs uninfected control cells, whereas steady-state levels of Mcl-1 protein were not increased by exposure to either H37Ra, HK bacteria, or LB (Fig. 2B). Similarly, in MDMs, there was a 2.5-fold increase of Mcl-1 protein measured in cells infected with H37Rv vs uninfected control cells, whereas there was no difference in the amount of Mcl-1 protein detected after exposure of cells to either H37Ra, HK bacteria, or LB (Fig. 2C).

Antisense oligonucleotides to *mcl-1* decrease Mcl-1 protein present in control and *M. tuberculosis*-infected macrophages

To determine whether Mcl-1 expression could be reduced in THP-1 cells and MDMs, phosphorothioate modified antisense and

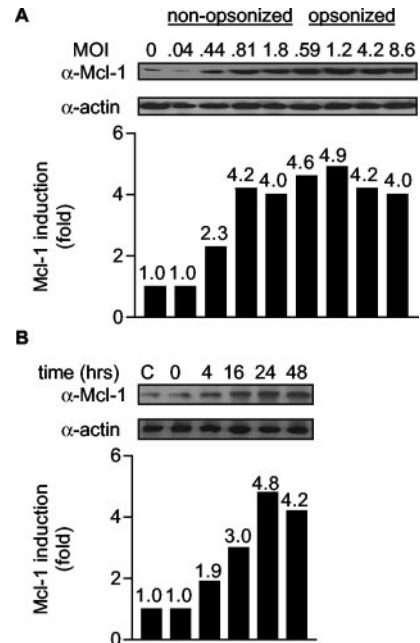


FIGURE 1. Mcl-1 protein is up-regulated in response to *M. tuberculosis* infection. **A**, THP-1 cells were uninfected (lane 1) or infected with non-opsonin-coated *M. tuberculosis* strain H37Rv at ratios of 5:1, 20:1, 50:1, or 200:1 (lanes 2–5) or with opsonin-coated *M. tuberculosis* strain H37Rv at ratios of 5:1, 20:1, 50:1, or 200:1 (lanes 6–9). Twenty-four hours after infection, whole cell lysates were separated by 12.5% SDS-PAGE and transferred to nitrocellulose for immunoblotting with anti-Mcl-1 Ab (upper blot) or anti-actin Ab (lower blot). **B**, THP-1 cells were uninfected (lane 1) or were infected with nonopsonin-coated *M. tuberculosis* strain H37Rv at a ratio of 50:1 and whole cell lysates were prepared immediately after infection (lane 2) or at 4, 16, 24, or 48 h after infection (lanes 3–6). Western blot analysis was performed as described in **A**. The blots shown are each from one experiment representative of two independent experiments with similar results. The graphs below each blot show the average fold increase in Mcl-1 protein expression normalized to actin compared with uninfected, control cells for the duplicate experiments.

sense oligonucleotides (s-oligos) were synthesized based upon sequences in human *mcl-1*. Incorporation of s-oligos into cells was verified by measuring the fluorescence of cells treated with FITC-labeled s-oligos compared with control cells by flow cytometry. FITC-labeled s-oligos were incorporated into 80–90% of cells in two independent experiments (data not shown). Treatment with antisense, but not sense, s-oligos decreased the amount of Mcl-1 protein in whole cell lysates of noninfected THP-1 cells by >90% and in noninfected MDMs by >96% (Fig. 3A). Mcl-1 protein was similarly reduced by 90% in whole cell lysates of THP-1 cells and MDMs infected with H37Ra and by 86% or 84%, respectively, in whole cell lysates of THP-1 cells or MDMs infected with H37Rv (Fig. 3B).

Apoptosis increases in *mcl-1* antisense s-oligo-treated macrophages infected with *M. tuberculosis* H37Rv

The contribution of Mcl-1 to THP-1 cell and MDM apoptosis during *M. tuberculosis* infection was examined by annexin V-PE binding to phosphatidylserine on the cell surface. Phosphatidylserine surface expression, an early marker of cell apoptosis, was assessed by annexin V-PE binding and flow cytometric analysis at 1 and 4 days after infection. One day after infection, compared with controls, the frequencies of annexin V-PE-positive cells were increased in response to infection with H37Ra but not H37Rv (Fig. 4, A and C). This effect was not influenced by treatment with *mcl-1*

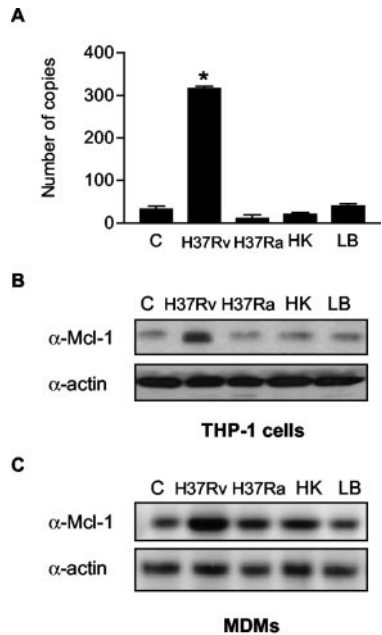


FIGURE 2. Infection with live *M. tuberculosis* strain H37Rv, but not exposure to strain H37Ra, HK, or LB causes an increase in *mcl-1* gene expression and Mcl-1 protein. **A**, Q-PCR was performed on reverse-transcribed total RNA prepared from differentiated THP-1 cells that were uninfected (C) or infected with *M. tuberculosis* strain H37Rv, strain H37Ra, HK, or LB at a 50:1 ratio. **B** and **C**, whole cell lysates of similarly treated THP-1 cells or MDMs were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Mcl-1 Ab (upper blots) or anti-actin Ab (lower blots). The blots shown are from one experiment representative of two independent experiments with similar results.

antisense. One day after infection with H37Rv, the percentages of annexin V-PE-positive THP-1 cells were similar in untreated and sense s-oligo-treated cells at $12 \pm 1\%$ and $15 \pm 1\%$, respectively. However, the percentage of H37Rv-infected cells that were positive for annexin V-PE binding increased dramatically to $29 \pm 4\%$ in THP-1 cells that were treated with antisense s-oligos to *mcl-1* (Fig. 4A, left graph). Pretreatment of cells with antisense s-oligos to *mcl-1* brought about a similar increase in annexin V-PE binding positive cells at 4 days postinfection with H37Rv (Fig. 4A, right graph). *M. tuberculosis* strain H37Rv-infected cells treated with sense s-oligos were $28 \pm 2\%$ annexin V-PE binding positive, whereas antisense s-oligo treatment increased this to $53 \pm 6\%$ positive cells (Fig. 4A, right graph). Whereas annexin V binding was again higher in H37Ra-infected cells, this time at 4 days after infection (Fig. 4A, right graph), as had been observed at 1 day postinfection, this response was not affected by antisense *mcl-1* treatment.

Qualitatively similar results were observed with annexin V-PE binding to untreated and sense- and antisense-treated MDMs that were noninfected or infected with *M. tuberculosis* strains H37Ra and H37Rv 1 and 4 days after infection (Fig. 4C, left and right graphs, respectively), although infection caused a larger amount of annexin V-PE binding in these cells. Infection with strain H37Ra caused significantly more annexin V-PE binding than did infection with strain H37Rv. Furthermore, antisense oligonucleotides to *mcl-1* caused an increase in annexin V-PE binding positivity in H37Rv-infected cells, but not in control cells or H37Ra-infected cells at 1 and 4 days after infection (Fig. 4C).

To ensure that the cause of the annexin V-PE binding to cells was apoptosis, we evaluated the number of treated THP-1 cells displaying nuclear fragmentation by TUNEL assay at 1 and 4 days

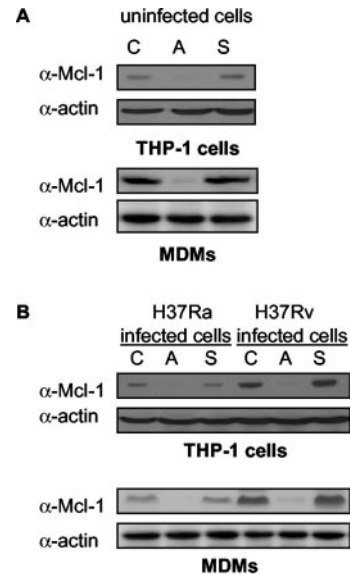


FIGURE 3. Antisense oligonucleotides to *mcl-1* decrease Mcl-1 protein present in uninfected or *M. tuberculosis*-infected THP-1 cells and MDMs. Cells were untreated (control) or were treated with antisense or sense s-oligos to *mcl-1* before differentiation with PMA. Cells were not infected (A) or infected with *M. tuberculosis* strain H37Ra or H37Rv (B). Twenty-four hours after infection, whole cell lysates were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Mcl-1 Ab (upper blots) or anti-actin Ab (lower blots). The blots shown are from one experiment representative of two independent experiments with similar results.

after treatment and infection (Fig. 4B). One day after infection with H37Rv, the percentages of TUNEL-positive THP-1 cells were similar in untreated and sense s-oligo-treated cells at $7 \pm 1\%$ and $8 \pm 1\%$, respectively. However, the percentage of H37Rv-infected cells that were TUNEL positive increased dramatically to $21 \pm 2\%$ in THP-1 cells that were treated with antisense s-oligos to *mcl-1* (Fig. 4B, left graph). Pretreatment of cells with antisense s-oligos to *mcl-1* caused a similar increase in TUNEL-positive cells 4 days after infection with H37Rv (Fig. 4B, right graph). *M. tuberculosis* strain H37Rv-infected cells treated with sense s-oligos were $17 \pm 2\%$ annexin V-PE binding positive, whereas antisense s-oligo treatment increased this to $36 \pm 3\%$ positive cells (Fig. 4B, right graph). Although TUNEL positivity was again higher in H37Ra-infected cells at 4 days after infection (Fig. 4B, right graph), as had been observed at 1 day postinfection, this response was not affected by antisense *mcl-1* treatment.

Mcl-1 induction in H37Rv-infected cells did not protect host cells from apoptosis induced by FasL or by ATP (data not shown). THP-1 cells were noninfected or infected with H37Rv or H37Ra followed by treatment with either 20 U/ml FasL or 20 mM ATP to induce apoptosis. Apoptosis was then measured by annexin V-PE binding to the surface of the cell membrane. FasL treatment resulted in induction of apoptosis in $\sim 72\%$ of cells after 24 h and this was not significantly different in noninfected cells, H37Rv-infected cells, or H37Ra-infected cells. ATP resulted in annexin V-PE binding in $>84\%$ of cells after 24 h and this also was not significantly different in noninfected, H37Rv-infected, or H37Ra-infected cells.

Attenuation of Mcl-1 expression reduces intracellular survival of *M. tuberculosis* strain H37Rv

To investigate whether *M. tuberculosis* strain H37Rv-induced Mcl-1 expression correlated with the intracellular survival of bacteria, growth of strains H37Ra and H37Rv was evaluated in THP-1

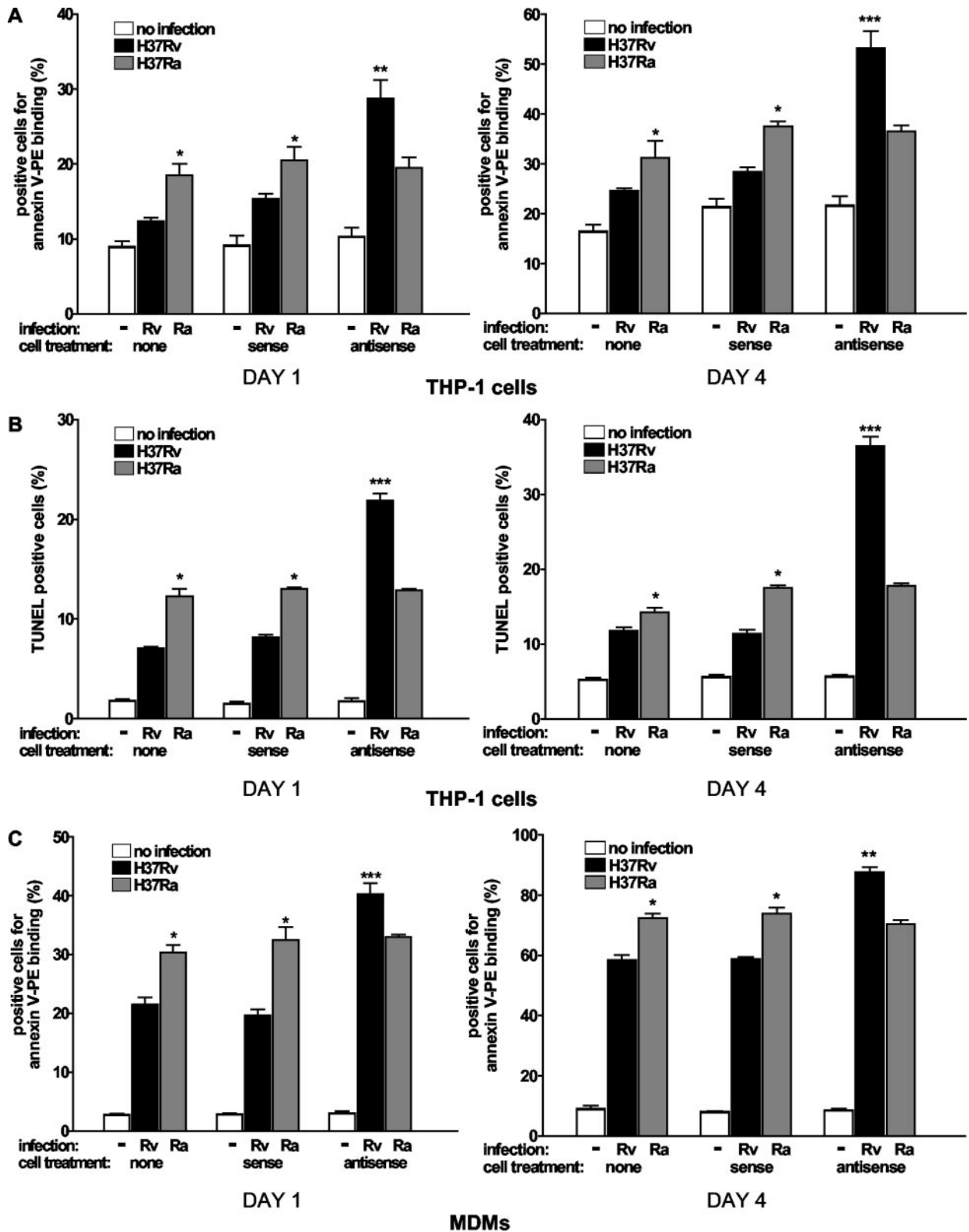


FIGURE 4. Antisense oligonucleotides to *mcl-1* increased apoptosis of H37Rv-infected macrophages 1 day (A) and 4 days (B) after infection. Cells were left untreated (control) or were treated with sense or antisense *s*-oligos to *mcl-1* before differentiation with PMA. Cells were then left uninfected (□) or infected with *M. tuberculosis* strain H37Rv (■) or strain H37Ra (▣). A and C, Annexin V-PE binding positive THP-1 cells or MDMs were measured by flow cytometry using untreated and uninfected cells as a negative control and actinomycin D-treated (0.5 μg/ml for 19 h) THP-1 cells as a positive control. B, TUNEL-positive THP-1 cells were measured by flow cytometry using untreated and noninfected THP-1 cells as a negative control and actinomycin D-treated cells as a positive control. Values represent the means ± SD from three independent experiments counting 10,000 events per treatment group in each experiment. *, $p < 0.05$ for H37Ra-infected cells untreated or treated with sense *s*-oligos compared with H37Rv-infected cells with the same treatments; **, $p < 0.01$; ***, $p < 0.005$ for H37Rv-infected and antisense *s*-oligo-treated vs H37Rv-infected and control, untreated, or sense *s*-oligo-treated cells and vs H37Ra-infected and antisense *s*-oligo-treated cells.

cells (Fig. 5A) and MDMs (Fig. 5B) that were left untreated or treated with sense or antisense s-oligos to *mcl-1*. Intracellular growth of H37Ra was similar whether cells were left untreated or treated with sense or antisense s-oligos to *mcl-1*. Growth of strain H37Rv was similar in untreated cells or cells treated with sense s-oligos to *mcl-1*. However, there were dramatic decreases in CFUs recovered from antisense s-oligo-treated cells at both 4 and 7 days after infection with H37Rv compared with cells treated with sense s-oligos (Fig. 5). Recovery of CFUs from antisense-treated

THP-1 cells was $42 \pm 3\%$ of CFUs recovered from sense s-oligo-treated, H37Rv-infected cells at 4 days after infection, and this was reduced further to only $21 \pm 2\%$ at 7 days after infection (Fig. 5A). Recovery of CFUs from antisense-treated MDMs was $56 \pm 4\%$ of CFUs recovered from sense s-oligo-treated, H37Rv-infected cells at 4 days after infection, and this was reduced further to only $19 \pm 2\%$ by 7 days after infection (Fig. 5B).

Discussion

This study examined changes in the expression of Bcl-2 family member Mcl-1 during infection with *M. tuberculosis* and the role of these changes to host cell apoptosis and to intracellular survival of bacteria. Intracellular infection with various species of mycobacteria has been reported to cause host cell apoptosis (33). Furthermore, infection with relatively avirulent species and strains such as *M. tuberculosis* H37Ra, *M. bovis* BCG, and *Mycobacterium kansasii* have been observed to induce more apoptosis than their more virulent counterparts including *M. tuberculosis* H37Rv, *M. bovis*, and a clinical *M. tuberculosis* isolate (5). This implies a role for apoptosis in host defense against the infection. Indeed, in infection models, apoptosis of the host macrophage has been shown to reduce the viability of intracellular *M. tuberculosis* (8–10).

The results presented in Fig. 1A demonstrate that induction of Mcl-1 by infection with virulent *M. tuberculosis* strain H37Rv occurred whether or not bacteria were serum opsonized (Fig. 1A). This suggests that Mcl-1 induction is independent of the route of entry of the bacilli. It has been reported that at least seven different receptors including CR1, CR3, CR4, mannose receptor, scavenger receptors, CD14, and sp-A are involved in the entry of *M. tuberculosis* into macrophages (34). Different receptor pathways may be favored by serum opsonization, but Mcl-1 induction occurs in the presence or absence of serum opsonins.

It has been observed that low MOIs for *M. tuberculosis* cause less apoptosis than high MOIs and in contrast that production of TNF- α was inversely proportional to MOI (35). Whereas this may represent a mechanism by which *M. tuberculosis* reduces host cell apoptosis and replicates within the host macrophage, we observed Mcl-1 induction to occur over a wide range of MOIs. Mcl-1 induction was evident at a MOI as low as 0.44 and increased further up to a MOI of 8.6, suggesting that induction of this antiapoptotic mechanism does not require low MOIs (Fig. 1A). Optimal induction of Mcl-1 occurred at 24 h after infection (Fig. 1B), suggesting that this mechanism is not triggered by phagocytosis per se, but rather by something elaborated by internalized bacteria.

In this study, H37Rv infection induced *mcl-1* gene expression (Fig. 2A) and Mcl-1 protein expression (Fig. 2, B and C), an antiapoptotic gene product, but H37Ra does not. This is consistent with a model in which host cell apoptosis provides an antibacterial defense mechanism that is subverted by virulent strain H37Rv (4, 5, 36). Mcl-1 induction did not occur after infection with H37Ra or phagocytosis of HK or LB, verifying that the response is not triggered by phagocytosis (Fig. 2), but rather infection with viable, virulent *M. tuberculosis* is required for induction. Taken together with the finding that maximal induction of Mcl-1 occurred between 24 and 48 h after infection, these results suggest that the induction of Mcl-1 is dependent upon a factor elaborated by viable and virulent *M. tuberculosis* within the phagocyte.

Antisense oligonucleotides to *mcl-1* reduced the expression of Mcl-1 protein in control cells (Fig. 3A) and in cells infected with either *M. tuberculosis* strain H37Ra or strain H37Rv (Fig. 3B). Mcl-1 expression in antisense s-oligo-treated, H37Rv-infected

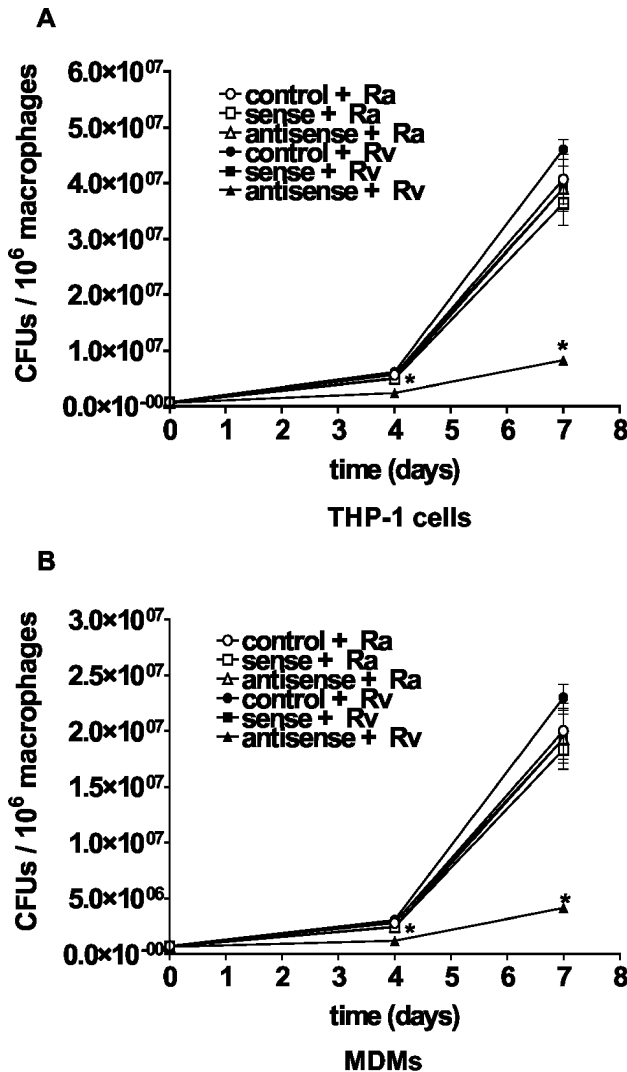


FIGURE 5. Antisense oligonucleotides to *mcl-1* reduce intracellular growth of *M. tuberculosis* strain H37Rv but not H37Ra. **A**, CFUs were recovered from THP-1 cells. **B**, CFUs were recovered from MDMs. CFUs were recovered from 10^6 untreated THP-1 cells (○, ●) or from sense (□, ■) or antisense (△, ▲) s-oligo-treated cells that were infected with either *M. tuberculosis* strain H37Ra (○, □, △) or H37Rv (●, ■, ▲). Macrophages were lysed with cold PBS containing 0.1% Triton X-100 immediately after infection or at 4 or 7 days after infection. *M. tuberculosis* was serially diluted in Middlebrook 7H9 with OADC and CFUs were determined by plating serial dilutions on Middlebrook 7H10 agar with OADC. CFUs were counted after 2 wk of incubation at 37°C. CFUs recovered immediately after infection were similar for all treatment groups. Values are the means and SD from three independent experiments plated in triplicate. *, $p < 0.02$ for H37Rv-infected and antisense-treated cells compared with H37Rv-infected and sense-treated cells. CFUs recovered from sense-treated cells infected with H37Rv and sense- or antisense-treated cells infected with H37Ra were not significantly different from those recovered from infection of untreated cells.

cells was lower than basal expression in untreated, uninduced control cells (Fig. 3B, lane 5 vs lane 1). The magnitude of the reduction in Mcl-1 expression was similar to that observed previously using the same antisense oligonucleotide sequence (26) where *mcl-1* antisense treatment resulted in an increase in apoptosis in polymorphonuclear cells induced by hypoxia while having no effect on control cells (26). Treatment with antisense s-oligos to *mcl-1* also resulted in an increase in apoptotic host cells in response to infection with H37Rv (Fig. 4). This suggested that Mcl-1 induction by virulent bacteria is an antiapoptotic mechanism. In contrast, for the attenuated strain H37Ra, which did not induce Mcl-1 expression, antisense treatment did not influence the level of apoptosis observed (Fig. 4).

Suppression of apoptosis by strain H37Rv may provide a means for increased intracellular bacterial proliferation. Inhibition of *mcl-1* expression by treatment with antisense s-oligos resulted in a reduced ability of H37Rv to grow within differentiated THP-1 cells and MDMs, respectively (Fig. 5). The induction of apoptosis in antisense-treated, H37Rv-infected THP-1 cells (Fig. 4, A and B) correlated with reduced recovery of CFUs from infected cells (Fig. 5A). Similarly, the annexin V-PE binding of antisense s-oligo-treated, H37Rv-infected MDMs (Fig. 4C) correlated with reduced recovery of CFUs from infected MDMs (Fig. 5B). This finding is consistent with a growing appreciation that apoptosis of infected macrophages leads to decreased survival of *M. tuberculosis*. For example, Molloy et al. (8) first described the antimycobacterial effect of host apoptosis in ATP-treated macrophages compared with the lack of antimycobacterial activity of necrotic macrophages (hydrogen peroxide treated). In addition, FasL-induced apoptosis had an antimycobacterial effect in vitro against *M. tuberculosis* strains H37Ra and H37Rv as did TNF- α -induced apoptosis (10). Recently, the antimycobacterial activity of picolinic acid against *M. avium* was attributed to induction of host cell apoptosis (9).

Although the increase in apoptosis in antisense oligonucleotide, H37Rv-infected THP-1 cells and MDMs correlated with a decrease in intracellular proliferation of this strain, the amount of cellular apoptosis did not correlate with intracellular growth throughout this study. For example, H37Ra infection was observed consistently to cause more host cell apoptosis than strain H37Rv (Fig. 4), yet its growth in untreated cells or sense oligonucleotide-treated cells was comparable to that of strain H37Rv. Hence, it may be that there is a threshold amount of apoptosis required to negatively effect intracellular growth of H37Rv. Alternatively, there may be mechanisms in addition to apoptosis that are negatively regulated by Mcl-1 which contribute to the reduced recovery of H37Rv when they are released from the inhibitory control of Mcl-1.

Attenuation of host cell apoptosis via induction of the antiapoptotic mechanisms by intracellular pathogens may provide a means to evade the host response, allowing increased bacterial proliferation (37). One example of a mycobacterial antiapoptotic mechanism is reduced Fas expression during *M. tuberculosis* infection, which may provide a means for *M. tuberculosis* to limit FasL-mediated apoptosis (10), promoting intracellular survival. A second example may relate to inhibition of TNF- α -induced apoptosis by virulent *M. tuberculosis*. Despite the fact that infection of macrophages with strains H37Rv or H37Ra leads to similar levels of TNF- α production, *M. tuberculosis* strain H37Ra causes more apoptosis than does strain H37Rv. This paradox may be explained by the finding that infection with *M. tuberculosis* strain H37Rv brings about greater expression of soluble TNFR 2 (sTNFR2), thereby blocking the activity of TNF- α (38). It has been proposed, therefore, that expression of sTNFR2 provides strain H37Rv with a means to decrease host cell apoptosis, thereby increasing its intra-

cellular survival. Increased expression of sTNFR2 by virulent strain H37Rv, but not attenuated strain H37Ra is consistent with the observation that avirulent strains of mycobacteria cause more apoptosis in host macrophages than virulent strains. Furthermore, the association of more host cell apoptosis with avirulent strains suggests that macrophage apoptosis is a host defense mechanism used to prevent proliferation of internalized bacilli (4, 5, 36). In this study, infection with *M. tuberculosis* strain H37Ra promoted significantly more macrophage apoptosis than was found during infection with strain H37Rv or in uninfected control cells 1 and 4 days after infection (Fig. 4). This observation correlates with the strain's inability to induce Mcl-1 expression and provides an additional mechanism whereby virulent strains of mycobacteria may cause less apoptosis than avirulent strains.

Mcl-1 induction may be a direct effect of signaling from *M. tuberculosis*. Alternatively, it may be an indirect effect of *M. tuberculosis* infection-induced cytokine production or a combination of both. It has recently been shown that TNF- α signaling in human prostate cancer cells leads to antiapoptotic *bcl-2* gene expression and *bcl-2* protein expression via NF- κ B (39). At present, although there has not been any direct evidence presented to link induction of other *bcl-2* family members to TNF- α signaling or NF- κ B activation, it is conceivable that other family members may be similarly regulated. Induction of another antiapoptotic *Bcl-2* family member, A1 protein encoded by the gene *bfl-1*, has recently been reported during infection of murine macrophages with live or dead *M. bovis* BCG (18). A1 induction was associated with the protection of host macrophages from NO-induced apoptosis. Mcl-1 induction did not protect H37Rv-infected macrophages from either FasL or ATP-induced apoptosis. The role of A1 in intracellular survival of BCG was not assessed (18).

Virulent mycobacteria use multiple, distinct strategies to prevent host cell apoptosis. Infection with virulent strains has been shown to prevent apoptosis by minimizing external signals that induce host cell apoptosis. Two such strategies have been demonstrated thus far including the decrease in Fas expression, which reduces FasL-induced apoptosis (10), and the increase in sTNFR expression, which competes for binding of TNF- α with the membrane receptor, thereby reducing TNF- α -mediated host cell apoptosis (38). Infection with virulent *M. tuberculosis* also increases expression of antiapoptotic proteins and this effect may be a direct effect of *M. tuberculosis* or may be an indirect effect of *M. tuberculosis* infection-induced cytokine production. Antiapoptotic genes induced after infection with virulent *M. tuberculosis* include *bcl-x_L*, *bfl-1*, and *mcl-1*, as demonstrated here (17, 18).

In this study, Mcl-1 was induced by infection of THP-1 cells with viable and virulent *M. tuberculosis*, but not in response to phagocytosis or ingestion of heat-killed or attenuated *M. tuberculosis*. Induction of this antiapoptotic protein limited the extent of apoptosis in virulent *M. tuberculosis*-infected macrophages as inhibition of Mcl-1 expression resulted in increased apoptosis of infected cells. This mechanism appears to contribute to the differential amounts of apoptosis observed when comparing virulent and avirulent mycobacterial strains and species. Finally, the induction of antiapoptotic Mcl-1 is important for the intracellular survival and/or proliferation of virulent *M. tuberculosis* as reduction of Mcl-1 expression by antisense oligonucleotides resulted in decreased CFUs recovered from H37Rv-infected macrophages. An important objective at this stage is to determine the nature of the factor produced during infection with viable and virulent strain H37Rv that leads to Mcl-1 induction. The evidence suggests that this factor must be either actively released or synthesized intracellularly since Mcl-1 induction was not observed in response to internalization of either dead bacilli or attenuated strain H37Ra. In

summary, these findings present a novel mechanism by which virulent *M. tuberculosis* evades apoptosis, an innate, protective host cell response to infection, thereby promoting its intracellular survival and proliferation.

Acknowledgments

We thank Drs. Vince Duronio and Kathryn Schubert for their valuable advice and technical assistance.

References

- Fenton, M. J. 1998. Macrophages and tuberculosis. *Curr. Opin. Hematol.* 5:72.
- Dolin, P. J., M. C. Raviglione, and A. Kochi. 1994. Global tuberculosis incidence and mortality during 1990–2000. *Bull. W. H. O.* 72:213.
- Santucci, M. B., M. Amicosante, R. Cicconi, C. Montesano, M. Casarini, S. Giosue, A. Bisetti, V. Colizzi, and M. Fraziano. 2000. *Mycobacterium tuberculosis*-induced apoptosis in monocytes/macrophages: early membrane modifications and intracellular mycobacterial viability. *J. Infect. Dis.* 181:1506.
- Keane, J., M. K. Balcewicz-Sablinska, H. G. Remold, G. L. Chupp, B. B. Meek, M. J. Fenton, and H. Kornfeld. 1997. Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. *Infect. Immun.* 65:298.
- Keane, J., H. G. Remold, and H. Kornfeld. 2000. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J. Immunol.* 164:2016.
- Placido, R., G. Mancino, A. Amendola, F. Mariani, S. Vendetti, M. Piacentini, A. Sanduzzi, M. L. Bocchino, M. Zembala, and V. Colizzi. 1997. Apoptosis of human monocytes/macrophages in *Mycobacterium tuberculosis* infection. *J. Pathol.* 181:31.
- Cree, I. A., S. Nurbhai, G. Milne, and J. S. Beck. 1987. Cell death in granulomata: the role of apoptosis. *J. Clin. Pathol.* 40:1314.
- Molloy, A., P. Laochumroonvorapong, and G. Kaplan. 1994. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guérin. *J. Exp. Med.* 180:1499.
- Pais, T. F., and R. Appelberg. 2000. Macrophage control of mycobacterial growth induced by picolinic acid is dependent on host cell apoptosis. *J. Immunol.* 164:389.
- Oddo, M., T. Renno, A. Attinger, T. Bakker, H. R. MacDonald, and P. R. A. Meylan. 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *J. Immunol.* 160:5448.
- Stenger, S., R. J. Mazzaccaro, K. Uyemura, S. Cho, P. F. Barnes, J. P. Rosat, A. Sette, M. B. Brenner, S. A. Porcelli, B. R. Bloom, and R. L. Modlin. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science* 276:1684.
- Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, et al. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* 282:121.
- Fairbairn, I. P., C. B. Stober, D. S. Kumararatne, and D. A. Lammas. 2001. ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X₇-dependent process inducing bacterial death by phagosome-lysosome fusion. *J. Immunol.* 167:3300.
- Stober, C. B., D. A. Lammas, C. M. Li, D. S. Kumararatne, S. L. Lightman, and C. A. McArdle. 2001. ATP-mediated killing of *Mycobacterium bovis* bacille Calmette-Guérin within human macrophages is calcium dependent and associated with the acidification of mycobacteria-containing phagosomes. *J. Immunol.* 166:6276.
- Kusner, D. J., and J. A. Barton. 2001. ATP stimulates human macrophages to kill intracellular virulent *Mycobacterium tuberculosis* via calcium-dependent phagosome-lysosome fusion. *J. Immunol.* 167:3308.
- Adams, J. M., and S. Cory. 2001. Life-or-death decisions by the Bcl-2 protein family. *Trends Biochem. Sci.* 26:61.
- Klingler, K., K. M. Tchou-Wong, O. Brandli, C. Aston, R. Kim, C. Chi, and W. N. Rom. 1997. Effects of mycobacteria on regulation of apoptosis in mononuclear phagocytes. *Infect. Immun.* 65:5272.
- Kausalya, S., R. Somogyi, A. Orlofsky, and M. B. Prystowsky. 2001. Requirement of A1-a for bacillus Calmette-Guérin-mediated protection of macrophages against nitric oxide-induced apoptosis. *J. Immunol.* 166:4721.
- Perskvist, N., M. Long, O. Stendahl, and L. Zheng. 2002. *Mycobacterium tuberculosis* promotes apoptosis in human neutrophils by activating caspase-3 and altering expression of Bax/Bcl-x_L via an oxygen-dependent pathway. *J. Immunol.* 168:6358.
- Mogga, S., T. Mustafa, L. Sviland, and R. Nilsen. 2002. Increased Bcl-2 and reduced Bax expression in infected macrophages in slowly progressive primary murine *Mycobacterium tuberculosis* infection. *Scand. J. Immunol.* 56:383.
- Harris, M. H., and C. B. Thompson. 2000. The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. *Cell Death Differ.* 7:1182.
- Shimizu, S., A. Konishi, T. Kodama, and Y. Tsujimoto. 2000. BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. *Proc. Natl. Acad. Sci. USA* 97:3100.
- Kozopas, K. M., T. Yang, H. L. Buchan, P. Zhou, and R. W. Craig. 1993. *MCL1*, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc. Natl. Acad. Sci. USA* 90:3516.
- Lomo, J., E. B. Smeland, S. Krajewski, J. C. Reed, and H. K. Blomhoff. 1996. Expression of the Bcl-2 homologue Mcl-1 correlates with survival of peripheral blood B lymphocytes. *Cancer Res.* 56:40.
- Druihl, A., M. Arock, L. Le Goff, and M. Pretolani. 1998. Human eosinophils express bcl-2 family proteins: modulation of Mcl-1 expression by IFN- γ . *Am. J. Respir. Cell Mol. Biol.* 18:315.
- Leuenroth, S. J., P. S. Grutkoski, A. Ayala, and H. H. Simms. 2000. The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis. *J. Leukocyte Biol.* 68:158.
- Liu, M. K., P. Herrera-Velitz, R. W. Brownsey, and N. E. Reiner. 1994. CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. *J. Immunol.* 153:2642.
- Stokes, R. W., and D. Doxsee. 1999. The receptor-mediated uptake, survival, replication, and drug sensitivity of *Mycobacterium tuberculosis* within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages. *Cell. Immunol.* 197:1.
- Sly, L. M., M. Lopez, W. M. Nauseef, and N. E. Reiner. 2001. 1 α ,25-Dihydroxyvitamin D₃-induced monocyte antimicrobial activity is regulated by phosphatidylinositol 3-kinase and mediated by the NADPH-dependent phagocyte oxidase. *J. Biol. Chem.* 276:35482.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
- Nandan, D., and N. E. Reiner. 1995. Attenuation of γ interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with *Leishmania donovani*: selective inhibition of signaling through Janus kinases and Stat1. *Infect. Immun.* 63:4495.
- Crowle, A. J., E. J. Ross, and M. H. May. 1987. Inhibition by 1,25(OH)₂-vitamin D₃ of the multiplication of virulent tubercle bacilli in cultured human macrophages. *Infect. Immun.* 55:2945.
- Kornfeld, H., G. Mancino, and V. Colizzi. 1999. The role of macrophage cell death in tuberculosis. *Cell Death Differ.* 6:71.
- Ernst, J. D. 1998. Macrophage receptors for *Mycobacterium tuberculosis*. *Infect. Immun.* 66:1277.
- Durrbaum-Landmann, I., J. Gercken, H. D. Flad, and M. Ernst. 1996. Effect of in vitro infection of human monocytes with low numbers of *Mycobacterium tuberculosis* bacteria on monocyte apoptosis. *Infect. Immun.* 64:5384.
- Fratuzzi, C., R. D. Arbeit, C. Carini, and H. G. Remold. 1997. Programmed cell death of *Mycobacterium avium* serovar 4-infected human macrophages prevents the mycobacteria from spreading and induces mycobacterial growth inhibition by freshly added, uninfected macrophages. *J. Immunol.* 158:4320.
- Weinrauch, Y., and A. Zychlinsky. 1999. The induction of apoptosis by bacterial pathogens. *Annu. Rev. Microbiol.* 53:155.
- Balcewicz-Sablinska, M. K., J. Keane, H. Kornfeld, and H. G. Remold. 1998. Pathogenic *Mycobacterium tuberculosis* evades apoptosis of host macrophages by release of TNF-R2, resulting in inactivation of TNF- α . *J. Immunol.* 161:2636.
- Catz, S. D., and J. L. Johnson. 2001. Transcriptional regulation of bcl-2 by nuclear factor κ B and its significance in prostate cancer. *Oncogene* 20:7342.