A Mechanism of Virulence: Virulent Mycobacterium tuberculosis Strain H37Rv, but Not Attenuated H37Ra, Causes Significant Mitochondrial Inner Membrane Disruption in Macrophages Leading to Necrosis

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A Mechanism of Virulence: Virulent *Mycobacterium tuberculosis* Strain H37Rv, but Not Attenuated H37Ra, Causes Significant Mitochondrial Inner Membrane Disruption in Macrophages Leading to Necrosis

Minjian Chen,*† Huixian Gan,* and Heinz G. Remold2*

Infection of human monocyte-derived macrophages with *Mycobacterium tuberculosis* at low multiplicities of infection leads 48–72 h after the infection to cell death with the characteristics of apoptosis or necrosis. Predominant induction of one or the other cell death modality depends on differences in mitochondrial membrane perturbation induced by attenuated and virulent strains. Infection of macrophages with the attenuated H37Ra or the virulent H37Rv causes mitochondrial outer membrane permeabilization characterized by cytochrome c release from the mitochondrial intermembrane space and apoptosis. Mitochondrial outer membrane permeabilization is transient, peaks 6 h after infection, and requires Ca\(^{2+}\) flux and B cell chronic lymphocytic leukemia/lymphoma 2-associated protein X translocation into mitochondria. In contrast, only the virulent H37Rv induces significant mitochondrial transmembrane potential (Δψ\(_{i}\)) loss caused by mitochondrial permeability transition. Dissipation of Δψ\(_{i}\) also peaks at 6 h after infection, is transient, is inhibited by the classical mitochondrial permeability transition inhibitor cyclosporine A, has a requirement for mitochondrial Ca\(^{2+}\) loading, and is independent of B cell chronic lymphocytic leukemia/lymphoma translocation into the mitochondria. Transient dissipation of Δψ\(_{i}\) 6 h after infection is essential for the induction of macrophage necrosis by *Mtb*, a mechanism that allows further dissemination of the pathogen and development of the disease. *The Journal of Immunology*, 2006, 176: 3707–3716.

Mitochondrial damage is of key importance in the outcome of *Mtb* infection with *Mtb* (5). In vitro infection of human Mø with attenuated *Mtb* induces predominantly apoptosis (6), a process that does not seem to be associated with the mitochondrial permeability transition (MPT)-dependent dissipation of mitochondrial transmembrane potential (Δψ\(_{i}\)) (7) and contains the pathogens within apoptotic bodies (8). In contrast, infection with virulent *Mtb* causes significant necrosis following irreversible MPT and leads to spread of the infection. These findings suggest that apoptosis and necrosis should be regarded as the extremes of a continuum.

The importance of apoptosis as a critical mechanism that protects against tuberculosis has been demonstrated recently in mice. In resistant mice, the supersusceptibility to tuberculosis 1 locus (*sst1*) leads to induction of Mφ apoptosis as a response to *Mtb* infection. In contrast, Mφ of *sst1* susceptible mice die after infection and show widespread necrosis (9). Thus, in vitro findings for human Mφ and in vivo findings for mice establish apoptosis of *Mtb*-infected Mφ as a central innate defense mechanism against tuberculosis.

In this study, we investigated the effects of attenuated and virulent *Mtb* on the integrity of the mitochondrial membranes of infected Mφ. Low multiplicity of infection (MOI) was used as occurs in human infection. We report that inoculation of human primary Mφ with both attenuated H37Ra and virulent H37Rv disrupts the mitochondrial outer membrane (MOM) (10–12), and that only the virulent H37Rv causes substantial loss of Δψ\(_{i}\), a consequence of MPT resulting in mitochondrial degradation and necrosis.

### Materials and Methods

#### Materials

Thapsigargin (TG), cyclosporin A (CsA), and ruthenium red (RR) were purchased from Sigma-Aldrich; 3,3′-dihexyloxy-carbocyanine iodide (*DIOC\(_{6}(3)\)*, rhodamine-2 AM, MitoTracker Green FM, Alexa Fluor 488, goat anti-mouse IgG1, and mouse anti-cyclooxygenase (COX) IV Ab were from...
Molecular Probes; mouse anti-cytochrome c mAb (clone 7H8.2C12, 6H2.B4) and mouse IgG1 were from BD Pharmingen; rabbit anti-annexin-I polyclonal Ab, goat anti-rabbit IgG FITC conjugate, and HRP-protein A were from Zymed Laboratories; murine anti-human actin mAb was from Pierce; rabbit anti-human B cell chronic lymphocytic leukemia/lymphoma 2-associated protein X (BaX) polyclonal Ab was from Santa Cruz Biotechnology; anti-phosphatidylserine mouse mAb (clone 1H6) and rabbit IgG were from Upstate Biotechnology; protease inhibitor mixture was from Roche; BaX small interfering RNA (siRNA) kit was from Cell Signaling Technology, and HEPES and DTT were from Invitrogen Life Technologies.

Quantification of mycobacteria
Adherent Mφ were inoculated with H37Ra or H37Rv at different MOI. After 4 h, the cells were washed five times with HBSS and cultured in IMDM. To measure mycobacterial growth, cells were lysed with 500 μl of 0.2% SDS in PBS. SDS was neutralized by addition of 500 μl of 50% FCS. A total of 100 μl of cell lysates from triplicate cultures was serially diluted 10-fold and plated on 7H10 agar plates (REMEL), and colonies were counted after 21 days. Alternatively, the cell lysates were pooled and inoculated in triplicate. For BaX visual estimation of mycobacterial growth was determined by use of the BacTec model 460TB system (BD Biosciences).

Cells and culture
Mononuclear cells from peripheral blood of healthy donors under informed consent and harvest under guidelines approved by the Brigham and Women’s Hospital Human Studies Committee were isolated, as previously described (5). Mφ were cultured for 7 days in IMDM (Invitrogen Life Technologies) containing 10% human AB serum (Gemini Bio-Products) and challenged with varying MOI of Mtb, as described.

In situ analysis of programmed cell death
Apoptosis of Mφ infected with virulent and attenuated Mtb was determined by use of a fluorescent in situ TUNEL assay (In Situ Cell Death Detection Kit; tetramethylrhodamine red; Roche) according to the manufacturer’s specifications. Necrosis was determined by counting the number of adherent cells at varying times after infection using an inverted phase contrast microscope (Nikon) equipped with a 10-mm2 grid in the eyepiece. Three wells per condition were counted at a magnification of ×100. All plates were assigned coded identification by an individual not involved in the study.

Necrosis of Mφ infected with virulent and attenuated Mtb was assessed using light microscopy of fixed and May–Grunwald-Giemsa-stained samples.

Flow cytometric analysis of mitochondrial cytochrome c release in Mtb (Mtb surface phosphatidylserine (PS) and annexin-I)
After diffusion of cytochrome c from the cytoplasm, Mφ with intact mitochondria stain positive for cytochrome c, while cells with mitochondria unable to retain mitochondrial cytochrome c stain negative (13). Mφ were cultured at 1.5 × 106 cells/ml in 6-well cluster plates (Corning Glass), washed with ice-cold PBS, and treated with 50 μg/ml digitonin in ice-cold PBS containing protease inhibitor mixture for 5 min on ice to allow selective permeabilization of the plasma membrane. After three washes with PBS, Mφ were fixed with 1% paraformaldehyde for 20 min at room temperature, dislodged from plates with a rubber policeman, pelleted at 500 × g, washed, and incubated in PBS containing 3% BSA and 0.05% saponin for 1 h. The cells were then incubated with mouse anti-cytochrome c mAb clone 6H2.B4 (BD Biosciences; 1/200 dilution) overnight at 4°C and with Alexa Fluor 488 goat anti-mouse IgG1 (1/200 dilution) for 1 h at room temperature, washed, resuspended in PBS containing 1% BSA, and analyzed under FL-1 logarithmic amplification using FACS. Mφ stained with irrelevant isotype Ab were used as controls.

For flow cytometric analysis of Mφ surface PS and annexin-I (14, 15), Mφ were washed twice with ice-cold PBS containing 1% BSA after fixation for 6 h, and incubated for 30 min on ice with mouse anti-PS monoclonal IgG (Upstate Biotechnology) or rabbit anti-annexin-I polyclonal Ab (Zymed Laboratories) at a dilution of 1/200 in FACS buffer. After washing with ice-cold PBS containing 1% BSA, cells were incubated for 20 min with goat anti-mouse Alexa Fluor 488 (Molecular Probes) for PS-FACS analysis, or goat anti-rabbit FITC conjugate (Zymed Laboratories) for annexin-I-FACS analysis. Cells were then fixed with 1% paraformaldehyde for 20 min. Green fluorescence was measured by FACS analysis. Mφ stained with irrelevant isotype Ab were used as controls.

Assessment of cytochrome c release from the mitochondria by Western blotting
Mφ were cultured at a density of 1.5 × 106 mononuclear cells/2 ml/well in 6-well cluster plates (Corning Glass) (5). The cells were infected with H37Rv at an MOI of 10:1 for 6, 12, and 48 h, washed with ice-cold PBS, and treated with 50 μg/ml digitonin in PBS in the presence of protease inhibitor mixture (Roche) for 5 min on ice. The digitonin solution was replaced with 500 μl of ice-cold extract buffer (250 mM sucrose, 20 mM HEPES (pH 7.5), 50 mM KCl, 2.5 mM MgCl2, 1 mM DTT, and protease inhibitor mixture). The cells were incubated for 20 min on ice, dislodged with a rubber policeman, and centrifuged at 1000 × g. A total of 20 μg of the supernatant protein was resolved in 15% SDS-PAGE gel. The polypeptides were transferred to polyvinylidene difluoride transfer membrane (PerkinElmer), treated with blocking buffer (5% nonfat dry milk in TBST), and incubated with mouse anti-cytochrome c mAb (clone 7H8.2C12, 1/500 dilution in blocking buffer). Actin was used as a loading control. After extensive washing with TBST, membranes were incubated with HRP-protein A at room temperature for 1 h, and the polypeptides were developed with Western Lighting Chemiluminescence (PerkinElmer Life Sciences) by exposure to x-ray films.

Assessment of MPT in Mφ
MPT was assessed in Mφ by evaluation of mitochondrial membrane potential (ΔΨm) dissipation by measuring retention of the lipophilic cationic dye DiOC6(3) within the mitochondria (16). Cells were preloaded with 1.5 nM DiOC6(3) in IMDM for 20 min at 37°C, washed, and incubated at 37°C for 10 min in medium containing 15 μg/ml digitonin, washed, and fixed with 1% paraformaldehyde for 20 min at 25°C. Cells were dislodged with a rubber policeman, pelleted at 500 × g, washed, and resuspended in PBS containing 1% paraformaldehyde. Flow cytometry to detect cells with diminished fluorescence was performed under FL-1 logarithmic amplification using FACS. Mφ stained with irrelevant isotype Ab were used as controls. In preliminary experiments, we tested whether fixation with paraformaldehyde alters the staining with the cationic dye DiOC6(3). After preloading with the dye, cells were fixed with 1% paraformaldehyde or not fixed before FACS analysis. No significant difference in DiOC6(3) fluorescence was detected.

Transfection of Mφ with BAX siRNA
Mφ were transfected with BAX siRNA using a BAX siRNA kit (Cell Signaling Technology), following the protocol of the manufacturer. Mφ plated at 1 × 106 cells/ml/well in 6-well cluster plates (Corning Glass) were cultured in IMDM with 10% human AB serum. Fresh medium was added to the cells 1 day before transfection. BAX siRNA or nontargeted siRNA (100 nM) were added to the cells. To determine RNA silencing of BAX expression, Mφ were transfected for 72 h, and the cells were then lysed in 1× SDS sample buffer. Cell lysates were fractionated on 15% SDS-PAGE using p42 as a loading control. To measure BAX translocation into mitochondria, infected Mφ were transfected and resuspended in PBS containing 500 μg/ml digitonin and protease inhibitor mixture for 5 min and centrifuged at 16 000 × g for 20 min. After washing, the pellets were re-suspended in 1× sample buffer, and 20 μg was resolved on 15% SDS-PAGE using COX IV as a loading control. To measure the release of DiOC6(3) release in siRNA-transfected Mφ after infection by FACS, the cells were transfected for 72 h, as described. The transfected cells in IMDM containing 2% human AB serum were inoculated with Mtb and were then prepared, as described for FACS analysis.

Confocal fluorescence imaging of intramitochondrial Ca2+ in Mtb
Mφ were cultured in 35-mm glass-bottom microwell dishes at a density of 1.5 × 106 mononuclear cells/2 ml/well, infected with 10 H37Ra/Cell for varying times, and then washed with IMDM. Cells were then preloaded with 2 μM dihydorothidamine-2-AM (prepared by reduction of rhodamine-2 AM following the manufacturer’s protocol) for 30 min at 4°C in IMDM containing 10% AB serum, incubated for 2 h at 37°C in IMDM without serum, and then loaded with 50 nM MitoTracker Green FM in IMDM without serum for 15 min. The cells were washed twice and resuspended in 500 μl of fresh medium, and confocal images of rhodamine-2 and MitoTracker Green were acquired with a Nikon TE-2000U laser-scanning confocal microscope using excitation wave lengths of 488 and 543 nm and emission wavelengths of 516 and 570 nm for MitoTracker Green and rhodamine-2,
respectively. All images were acquired at 37°C using a temperature-controlled chamber. Mitochondria were identified by the green fluorescence of the MitoTracker Green.

**Histology**

Mφ (2.5 × 10⁵/ml/well) were cultured on 12-mm-diameter coverslips (Propper Manufacturing) for 7 days to allow attachment. After infection with Mφ for 48 h, the cells were washed twice with IMDM and fixed in methanol:acetic acid (3:1 vol) for 24 h. The cells were stained with Wright’s stain (Fisher Scientific) for 10 min and Giemsa stain for 20 min. The cells were then examined by light microscopy and were photographed with a Leica DFC300 digital camera.

**Electron microscopy**

Mφ were cultured in IMDM containing 10% human AB serum in a 6-well plate. The cells were infected with H37Rv or H37Ra (MOI 10) for 48 h, fixed with 100 mM cacodylate buffer (1.25% formaldehyde, 2.5% glutaraldehyde, and 0.03% picric acid) overnight at 4°C, and postfixed in 1% osmium tetroxide containing 1.5% potassium ferrocyanide for 30 min. The cells were then stained with 1% uranyl acetate in maleate buffer (pH 5.2) for 30 min at room temperature. After dehydration, the cells were removed from the plate in propylene oxide and centrifuged at 3000 rpm. The cells were then examined by scanning electron microscopy (JEOL), and recorded on Kodak sheet film (Eastman Kodak).

**Statistics**

Results are expressed as mean ± SE. The data were analyzed by using Microsoft Excel Statistical Software (Jandel) using t test for normally distributed data with equal variances. A p value <0.05 was considered statistically significant.

**Results**

_Virulent H37Rv and attenuated H37Ra induce transient release of cytochrome c from the mitochondria in Mφ_

In the mitochondrial pathway of apoptosis, MOM permeabilization (MOMP) leads to release of proapoptotic factors, including apoptosis-inducing factor, Htr2/Omi, endo 6, Smac/Diablo, and cytochrome c from the mitochondrial intermembrane space into the cytosol, resulting in the activation of downstream caspases and apoptosis (see online supplement to Ref. 7). To assess the effect of virulent and attenuated Mφ on MOM MOMP, Mφ were inoculated with H37Ra or H37Rv, and cytochrome c release was measured by flow cytometry as a function of time. The number of cells with depleted mitochondrial cytochrome c is up-regulated at 6 h after infection (Fig. 1, A and B). At 12 and 24 h, the number of cytochrome c-depleted Mφ has almost returned to baseline levels, indicating that MOM permeability recovers. At 48 h, the number of cells with depleted mitochondrial cytochrome c increases again (Fig. 1, A and B), reflecting ΔΨm, dissipation associated with mitochondrial degradation and necrotic death (10).

In dose-response experiments, H37Ra and H37Rv at MOI of 5 minimally increased mitochondrial cytochrome c release at 6 h (Fig. 1C). At MOI 5, H37Rv produces more Mφ with depleted mitochondrial cytochrome c than H37Ra (p = 0.01), and at MOI 10 both strains generate comparable numbers of Mφ with depleted mitochondrial cytochrome c.

These results could be confirmed by Western blotting of cytochrome c translocated into the cytosol of H37Rv-infected Mφ. The cytochrome c concentration in the cytosol is increased at 6 and

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**FIGURE 1.** Release of mitochondrial cytochrome c in Mφ infected with attenuated Mφ strain H37Ra and virulent strain H37Rv. Mφ were inoculated with the indicated MOI, cultured for the indicated times (A, B, or for 6 h (C)), harvested, permeabilized, stained with cytochrome c Ab, and analyzed by FACS. A, Histograms show a representative experiment for Mφ infected with H37Ra at MOI 10 after staining with cytochrome c Ab (open profiles). The shaded profiles indicate Mφ stained with irrelevant isotype Ab. The gate to select Mφ with depleted cytochrome c is indicated by the bar on the top. B, Summary of three experiments for H37Ra ( ) and H37Rv ( ) at the indicated MOI. Mφ infected with H37Ra at MOI 5 ( ) and with H37Rv at MOI 10 ( ) were examined only at 6 and 48 h. The difference between the numbers of Mφ with depleted mitochondrial cytochrome c at 6 time and 6 h was statistically significant for H37Rv (p = 0.01) and H37Ra/MOI 10 (p = 0.02). Cytochrome c release at 6 h reflects MOMP; loss at 48 h reflects the mitochondrial degradation that precedes Mφ necrosis (detailed below). C, Dose response. Shown are mean values ± SE for four experiments for Mφ infected with H37Ra and H37Rv at the indicated MOI, harvested, and analyzed at 6 h. D, Mitochondrial cytochrome c translocation into the cytosol of Mφ infected with H37Rv (MOI 10) as determined by Western blotting is seen 6 and 48 h after infection. No extramitochondrial cytochrome c accumulation is detected 12 h after infection.
of the apoptotic markers PS and annexin-1 (14, 15, 17). Infection or for annexin-1, and examined by FACS. The difference in the increase of H37Ra and H37Rv (MOI 10), harvested after 6 and at 48 h, stained for PS and 12 and 24 h, the number of M* of M with augmented cell surface PS is not statistically different from untreated controls, when M* are infected with H37Rv, indicating that induction of apoptotic markers by the virulent H37Rv is attenuated when compared with infection with H37Ra (6). In contrast, infection of M* with H37Ra and with H37Rv increased levels of cell surface annexin-1 at 6 and 24 h after infection (Fig. 2). The increase of the numbers of M* with augmented surface annexin-1 is significantly smaller, when the cells are infected with H37Rv.

Virulent H37Rv, but not attenuated H37Ra, induces significant dissipation of $\Delta \psi_m$

We next investigated whether M* induces opening of the inner mitochondrial membrane. MPT pore opening associated with mitochondrial inner membrane perturbation is an independent event that frequently accompanies MOMP (7). When the permeability transition pore opens, collapse of the mitochondrial inner membrane potential $\Delta \psi_m$ ensues as a consequence of dissipation of the proton gradient generated in the mitochondrial intermembrane space. Opening of the permeability transition pore uncouples the respiratory chain and causes overproduction of superoxide anions (16, 18), leading to necrosis. To test whether virulent and attenuated M* strains differ in their capacity to induce $\Delta \psi_m$ dissipation, we infected M* with H37Ra and H37Rv. H37Ra was initially used at MOI 10 and H37Rv at MOI 5 to equalize cytochrome c release at 6 h. Transient MPT determined by FACS quantification of cells with depleted DiOC$_6$(3) is maximal for both M* strains at 6 h after infection (Fig. 3, A and B) and is significantly greater, when M* are infected with H37Rv than with H37Ra ($p = 0.01$) (Fig. 3B). At 12 and 24 h, the number of M* with depleted mitochondrial DiOC$_6$(3) has returned almost to baseline in both cultures, indicating that MPT at 6 h is transient and mitochondrial inner membrane impermeability has recovered. Dose-response studies indicate that H37Rv is ~4 times more effective as an inducer of MPT than H37Ra (Fig. 3C). At 36 and 48 h, H37Rv induces greater numbers of M* with depleted mitochondrial DiOC$_6$(3), a correlate of the necrotic state of the cells, than H37Ra ($p = 0.009$ at 36 h; $p = 0.018$ at 48 h). We next compared the dose response of H37Rv with respect to MPT and MOMP at 6 h. At low MOI (0.5 and 2), H37Rv induces significant MPT while leaving MOM intact (Fig. 3D). The difference of the number of M* with depleted mitochondrial cytochrome c or depleted mitochondrial DiOC$_6$(3) at 6 h is statistically significant at MOI 0.5 ($p = 0.01$) and MOI 2 ($p = 0.001$; Fig. 3D) and indicates that MPT at 6 h occurs in M* with intact MOM, and that these cells might proceed directly to undergo necrosis. MPT without MOMP was also reported in granuloma A-treated apoptotic K562 cells (19).

We further tested whether differential uptake of H37Ra and H37Rv by the M* accounts for differential induction of MPT and MOMP. To that end, we measured phagocytosis of H37Ra and H37Rv after 4 h of incubation of the M* with both strains at various MOI. The number of ingested bacteria was not found to be different for H37Ra and H37Rv (Fig. 3E).

Consistent with these results, M* infected with H37Rv undergo more necrosis than cultures infected with H37Ra, because M* are undergoing cytolyis in H37Rv-infected M* cultures than in cultures infected with H37Ra. As assessed by light microscopy 72 h after infection with H37Ra (MOI 10), the percentage of M* undergoing cytolyis was 36 + 14%. In contrast, M* cultures infected with H37Rv (MOI 10) contained at 72 h 82 + 5% M* undergoing cytolyis. Pretreatment of the H37Rv-infected M* cultures with CsA (5 μM) reduced the number of necrotic cells to 29 + 13%. The difference between these values is statistically significant ($p < 0.005, n = 3$).

With respect to this study, the important difference between apoptosis and necrosis is the lack of M* plasma membrane integrity associated with necrosis. In contrast, apoptotic M* have intact plasma membranes that prevent exocytosis of inflammatory components from the cytoplasm and allow containment of the pathogens within the cells. M* infected for 48 h with H37Ra (MOI 10) show signs of apoptosis such as pycnotic nuclei, but have intact plasma membranes. In contrast, M* infected with the virulent H37Rv show significant disruption of the plasma membrane (Fig. 4).

Because in some cells cell death induced by MOMP is abrogated by caspase inhibitors (20), we tested whether $\Delta \psi_m$ loss of H37Rv-infected M* is dependent on caspase activation. To that end, we treated M* with the pan-caspase inhibitor zVAD-fmk before inoculation. Caspase inhibition does not decrease $\Delta \psi_m$ loss in these cells. The number of M* with cationic dye release in absence of zVAD-fmk was 45.8 + 3.5% and in presence of the inhibitor 44.6 + 3.8% ($p = 0.1, n = 3$), indicating that induction of MOMP by M* is caspase independent.

$Ca^{2+}$ flux into the mitochondria is required for MOMP and MPT of M* infected M*

In many cell systems, increase of mitochondrial $Ca^{2+}$ levels is required for MOMP (21) and is also an important modulator of MPT (22). We therefore investigated whether after infection with M* mitochondrial $Ca^{2+}$ loading is important for the induction of MOMP and MPT. M* were preincubated with RR, an inhibitor of the mitochondrial $Ca^{2+}$ uniporter (23) that prevents mitochondrial calcium loading, and were inoculated with H37Ra and H37Rv, and MOMP and MPT were measured (Fig. 5A). Pretreatment with RR inhibits both mitochondrial cytochrome c release and release of DiOC$_6$(3) of H37Ra and H37Rv-infected M*, indicating that mitochondrial $Ca^{2+}$ loading is required for the induction of MOMP and of MPT. Addition of RR to uninfected cells has no effect. Furthermore, pretreatment of M* with TG, a sarcoplasmic/endoplasmic reticulum calcium ATPase pump inhibitor that causes passive $Ca^{2+}$ release from the endoplasmatic reticulum.
stores and concomitant increases of intramitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{m}\)) (24), augments MPT and MOMP. Treatment with TG to increase mitochondrial calcium loading augments exposure of the apoptotic markers PS and annexin-1 on the Mϕ surface, and RR treatment to decrease mitochondrial Ca\(^{2+}\) levels substantially diminishes surface expression of both apoptotic markers (data not shown).

To demonstrate that infection with \(Mtb\) indeed leads to Ca\(^{2+}\) influx into the mitochondria, we analyzed H37Ra-infected Mϕ by confocal fluorescence microscopy using rhodamine-2, a Ca\(^{2+}\)-sensitive fluorescent dye that preferentially accumulates in mitochondria (25) and MitoTracker Green to label the mitochondria (26).

This approach revealed an increase of [Ca\(^{2+}\)]\(_{m}\) at 6 h coincident with the peak of transient MOMP and Δφ\(_{m}\) loss (Fig. 5B, row 2) that is not found at 12 or 24 h (Fig. 5B). More than 90% of the Mϕ contained increased [Ca\(^{2+}\)]\(_{m}\) at 6 h (data not shown). TG (0.2 μM) further increased [Ca\(^{2+}\)]\(_{m}\) in H37Ra-infected Mϕ at 6 h, and RR abrogated the Ca\(^{2+}\) uptake into mitochondria induced by H37Ra.

**Translocation of BAX to the mitochondria is required for MOMP:** Δφ\(_{m}\) dissipation is independent of BAX translocation

Apoptosis is initiated by cleavage and activation of cytosolic BID by proteases that cause translocation of BAX into the mitochondria and activation of BAK, resulting in the release of cytochrome c.
into the cytosol and in apoptosis (27, 28). To test the role of BAX in Mtb-induced Mφ death, we examined the translocation of the cytoplasmic protein BAX into the mitochondria of infected Mφ. BAX was found to be associated with mitochondria at 3 h and at all later time points after inoculation with H37Ra or with H37Rv (Fig. 6A). BAX redistribution was not affected by changes in Ca2+ flux into the mitochondria (Fig. 6B). RR (23), an inhibitor, and TG, a promoter of Ca2+ flux into the mitochondria (24), do not alter BAX translocation.

Treatment of infected Mφ with BAX siRNA decreases BAX expression (Fig. 6C) and blocks mitochondrial cytochrome c release at 6 h (Fig. 6D), demonstrating that BAX translocation is required for MOMP. In contrast, abrogation of BAX expression does not inhibit mitochondrial DiOC6(3) depletion. MPT proceeds irrespective whether BAX is translocated into the mitochondria or not (Fig. 6E). This is not surprising, because BAX activation is a prerequisite for MOMP (28) and independent of MPT.

MOMP and MPT are associated with apoptosis and necrosis, respectively

We next performed experiments to investigate whether MOMP is associated with the induction of apoptosis. Infection of Mφ with H37Ra (MOI 10) induces MOMP 6 h after infection, and MPT induction is negligible (Fig. 3B). Up-regulation of cell surface exposure of the apoptotic markers PS and annexin-1 also indicates that MOMP and not MPT is associated with apoptosis (Fig. 2). At 48 h, the number of TUNEL-positive Mφ is also significantly increased under these infection conditions (Fig. 7A, left panel).

Treatment of Mφ with CsA (5 μM), a classic inhibitor of MPT, does not inhibit accumulation of TUNEL-positive Mφ (Fig. 7A, left panel), accumulation of Mφ with depleted mitochondrial cytochrome c at 6 h (Fig. 7B), and of Mφ with increased surface exposure of PS and annexin-1 (Fig. 7C). These findings indicate that MPT is independent of MOMP (7). In contrast, susceptibility of necrosis (Fig. 7A, right panel) and of mitochondrial DiOC6(3) depletion, an indicator of Δψm loss (Fig. 7D), to the effect of CsA shows that these events are caused by MPT (16).

When multiple Mtb infection studies are subjected to linear regression analysis, the extent of transient Δψm loss at 6 h correlates with the number of Mφ entering the necrotic state at 48 h (Fig. 8A), suggesting that up-regulation of early MPT commits the Mφ to necrosis at 48 h.

Up-regulation of the numbers of Mφ with depleted cytochrome c in comparison with the number of cells with cationic dye depletion at 6 h correlates with diminished numbers of prenecrotic cells at 48 h (Fig. 8B). These findings indicate a possible protective effect of MOMP. Alternatively, attenuated Mtb might induce

**FIGURE 4.** Morphology of Mφ infected with the attenuated H37Ra and the virulent H37Rv. Mφ infected with H37Ra and H37Rv for 48 h were processed for electron microscopical examination (see Materials and Methods). Upper panels, Pictures of single cells at a magnification of ×3,000; lower panels, sections of the plasma membrane of the same cell at a magnification of ×12,000. Note that H37Ra induces apoptosis characterized by nuclear pycnosis (upper center panel, arrow) and intactness of the plasma membrane (center panels). In contrast, H37Rv causes destruction of the plasma membrane (right panels). The arrow in the lower right panel indicates where the continuity of the plasma membrane is interrupted. An uninfected Mφ is seen in the panels on the left.
changes in the mitochondria that cause MPT accompanied by MOMP, favoring apoptosis, and virulent Mtb might increase MPT, but inhibit MOMP, leading to increased necrosis.

Discussion

The major findings of this study are that the virulent H37Rv induces significantly more MPT and Δψm dissipation than the attenuated H37Ra 6 h after infection of the Mφ. In contrast, both the attenuated Mtb strain H37Ra and the virulent strain H37Rv cause MOMP. Both MPT and MOMP at 6 h are transient, and the capacity of MOM to retain cytochrome c has largely recovered at 12 and 24 h after infection. Reversible MOMP is also induced in isolated THP-1 mitochondria, when incubated with cytosol from Mφ infected for 6 h, suggesting a role for cytosolic factors and not a direct effect of Mtb on the mitochondria in the induction of MOMP (data not shown). Induction of MOMP is associated with the appearance of the early apoptotic markers PS and annexin-1 on the Mφ surface. In contrast to MOMP, H37Rv-induced MPT at 6 h is blocked by CsA, a classic inhibitor of MPT. Using agents that specifically alter Ca²⁺ influx into the mitochondria, we show that both MPT and MOMP are dependent on mitochondrial calcium loading. As expected, inhibition of BAX activation blocks MOMP, but does not affect early transient Δψm dissipation. Moreover, the extent of transient Δψm dissipation in Mtb-infected Mφ at 6 h correlates with the number of necrotic cells 48 h after infection. Cumulatively, these findings indicate that Mtb induce MOMP and...
MPT by independent mechanisms, and that virulent Mtb induces significantly more MPT than attenuated bacilli.

MOMP causes the release from the mitochondria of caspase activators, including cytochrome c, that are normally sequestered between the inner and outer mitochondrial membrane (29) (see online supplement to Ref. 7). After release into the cytosol, cytochrome c forms a complex containing Apaf-1, ATP, and pro-caspase-9, resulting in the activation of downstream caspases (12) and apoptosis. Cytosolic cytochrome c also binds to inositol (1,4,5) triphosphate receptors of the endoplasmatic reticulum, amplifying the Ca\(^{2+}\) release from the endoplasmatic reticulum (30) that causes mitochondrial Ca\(^{2+}\) influx necessary for induction of apoptosis (31).

In contrast to apoptosis, necrosis is a consequence of the opening of the MPT pore (32). Components of this pore are adenine nucleotide translocase, the voltage-dependent anion transporter, and cyclophilin D, a small protein present inside the mitochondria that binds CsA and is involved in MPT (33). CsA prevents cyclophilin D binding to adenine nucleotide translocase, thereby inhibiting the opening of the MPT pore and MPT. In the case of infection with the virulent H37Rv when MPT is induced, irreversible mitochondrial damage ensues leading to necrosis and cytolysis of the affected cells. Thus, MPT is exploited by virulent Mtb to destroy the host cell, leading to spread of the infection.

An additional important mechanism that might be involved in the manifestation of apoptosis and might be suppressed by virulent Mtb is the activation of plasma membrane repair mechanisms required for repair of damaged plasma membranes. These mechanisms require transiently increased intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and use lysosomal membrane components to restore disrupted plasma membranes (34). Because necrosis is characterized by damage of the plasma membrane, we investigated whether increase of [Ca\(^{2+}\)]\(_i\), required for the activation of plasma membrane repair mechanisms blocks necrosis. Addition of the ionophore A23187 to M\(\phi\), which increases [Ca\(^{2+}\)]\(_i\), indeed down-regulates M\(\phi\)-induced necrosis (5), suggesting that Ca\(^{2+}\)-regulated repair mechanisms might be involved in maintaining the apoptotic state (35). This topic is currently under investigation in our laboratory.

There is a discrepancy between apoptosis and cytochrome c release, when the effects of avirulent and virulent Mtb strains are compared. Although apoptosis is more pronounced in H37Ra than in H37Rv-infected M\(\phi\) cultures, cytochrome c release is similar in H37Rv and in H37Ra-infected M\(\phi\) cultures. This paradox might be explained by the fact that in H37Rv-infected M\(\phi\) cultures a significant number of the apoptotic cells has become necrotic, leading to a decrease of apoptotic M\(\phi\) numbers in H37Rv-infected M\(\phi\) cultures. In contrast, in H37Ra-infected M\(\phi\) cultures, generation of necrotic cells is delayed and more apoptotic cells accumulate at any time point.

Several studies cite irreversible MPT as one of the key elements of caspase-dependent apoptosis (36, 37). Mitochondrial damage caused by MPT induces disruption of mitochondrial function by \(\Delta \psi_m\) loss and consequent increase of reactive oxygen species production. Our findings differentiate between MTP and MOMP as effector mechanisms in Mtb-induced M\(\phi\) death and corroborate findings of others that apoptosis is initiated in absence of mitochondrial permeability pore opening by MOMP (summarized in Ref. 7). MOMP is caspase and BAX dependent and does not affect the integrity of the mitochondria.

Furthermore, studies using mice with targeted disruption of cyclophilin D unable to undergo MPT and necrosis demonstrate that MOMP proceeds normally in these mice (38, 39). Cyclophilin D-deficient cells do not undergo CsA-sensitive mitochondrial membrane changes and are resistant to ischemia/reperfusion-induced necrosis, indicating that perturbation of the inner mitochondrial membrane regulates necrosis. Cyclophilin D deficiency does, however, not affect the release of cytochrome c from the mitochondria through MOMP, an event essential for the induction of apoptosis (39).

Recent findings suggest mechanisms that might partially explain the basis for MPT induction by virulent Mtb strains. Among the genes that are markedly down-regulated in the attenuated H37Ra in comparison with H37Rv are genes coding for members of the protein family containing 6-kDa early secretory Ag target (ESAT-6) (40), mycobacterial proteins that induce potent Th1 responses and elicit protection against tuberculosis (41) when administered as subunit or DNA vaccines (42). When bacillus Calmette-Guerin and Mycobacterium bovis were compared, the genes expressing ESAT-6 and culture filtrate protein-10 (exxA and exxB) were found to be part of the region of difference 1 (43). exxA and exxB are missing from the attenuated bacillus Calmette-Guerin, but are present in M. bovis and in virulent Mtb (44). ESAT-6 and culture-filtrated protein-10, which are secreted by the
bacilli as a tight 1:1 complex (45), can be pinocytosed by Mφ and could act directly on the mitochondrial inner membrane, or, alternatively, might induce a factor(s) that disrupts the mitochondrial inner membrane.

Because the apoptotic state of the Mφ has been linked to anti-mycobacterial activity (8) and enhanced Ag presentation (46), we propose that apoptosis of infected Mφ is an innate defense mechanism that reduces the intracellular burden of Mtb, whereas necrosis promotes spread of the infection by cytolysis of the host Mφ. Because <10 inhaled bacilli suffice to establish infection, our in vitro model with low MOI (<10) is particularly relevant to the early phase of Mtb exposure and development of human tuberculosis, indicating that the balance of apoptosis vs necrosis of infected Mφ determines the course of Mtb infection in vivo. This concept is also supported by a recent study in the murine system (9), which shows that a host gene (Ipr1) linked to tuberculosis concept is also supported by a recent study in the murine system.

The understanding of how the pathogens on the mitochondrial membranes and the Mtb inner membrane.

could act directly on the mitochondrial inner membrane, or, alter-

References

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caused by the inhibitor binding to mitochondrial matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem. J. 268: 153–160.


