Critical Role of Mitochondrial Damage in Determining Outcome of Macrophage Infection with *Mycobacterium tuberculosis*[1]

Lei Duan,[2]* Huixian Gan,[2]* David E. Golan,[†] and Heinz G. Remold[3]*

Human macrophages (Mϕ) respond to *Mycobacterium tuberculosis (Mtb)* infection by undergoing apoptosis, a cornerstone of effective antimycobacterial host defense. Virulent mycobacteria override this reaction by inducing necrosis leading to uncontrolled *Mtb* replication. Accordingly, Mϕ death induced by inoculation with *Mtb* had the characteristics of apoptosis and necrosis and correlated with moderate increase of mitochondrial permeability transition (MPT), mitochondrial cytochrome *c* release, and caspase-9 and -3 activation. We hypothesized that changes in intramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) determine whether Mϕ undergo either apoptosis or necrosis. Therefore, we induced mechanism(s) leading to predominant apoptosis or necrosis by modulating $[Ca^{2+}]_m$ and examined their physiological consequences. Adding calcium ionophore A23187 to Mϕ inoculated with *Mtb* further increased calcium flux into the cells which is thought to lead to increased $[Ca^{2+}]_m$, blocked necrosis, stabilized MPT, decreased mitochondrial cytochrome *c* release, lowered caspase activation, and accompanied effective antimycobacterial activity. In contrast, Mϕ infected with *Mtb* in presence of the mitochondrial calcium uniporter inhibitor ruthenium red showed increased mitochondrial swelling and cytochrome *c* release and decreased MPT and antimycobacterial activity. Thus, in *Mtb*-infected Mϕ, high levels of mitochondrial membrane integrity, low levels of caspase activation, and diminished mitochondrial cytochrome *c* release are hallmarks of apoptosis and effective antimycobacterial activity. In contrast, breakdown of mitochondrial membrane integrity and increased caspase activation are characteristic of necrosis and uncontrolled *Mtb* replication. ***The Journal of Immunology,*** **2002, 169: 5181–5187.**

Estimates indicate that one-third of the world's population is infected with *Mycobacterium tuberculosis (Mtb)*[4], the causative agent of tuberculosis (1). Tuberculosis persists as a major cause for morbidity and mortality throughout the world due to issues of cost, compliance with drug treatment, drug resistance, and the AIDS epidemic. Thus, there is urgent need for enhanced knowledge of disease pathogenesis that might be the basis for novel approaches to treat and prevent this disease. Whereas the pathology of the disease is relatively well understood (2), little is known of the front line antimycobacterial defense mechanisms.

In broad terms, we know that defense mechanisms of the innate immune system are of critical importance in the early suppression of the *Mtb* infection. It is known that macrophages (Mϕ), the first line of defense against tuberculosis, undergo apoptosis when inoculated with *Mtb* and develop significant antimycobacterial activity (3–5). *Mtb*-induced Mϕ apoptosis requires a dual signal, the action of TNF-α (6), and the activation of cytosolic phospholipase A_2 (7), and is thought to result in activation of caspases, including caspase-9 and caspase-3. Caspase-9 is an initiator caspase of apoptosis and its activation, which leads to activation of the effector caspase caspase-3, requires formation of the "apoptosome", a complex formed by cytochrome *c* released from the mitochondria into the cytoplasm, the cytosolic factor Apaf-1, ATP, and procaspase-9 (8, 9). Apoptosis is generally characterized by membrane blebbing, cellular condensation, and DNA fragmentation (10), and leads to removal of the sealed apoptotic bodies by phagocytosis predominantly through the phosphatidylserine receptor on the Mϕ (11).

In addition to apoptosis, there exists also an alternative cell death program, necrosis, which typically involves swelling of the cell and organelles, resulting in lysis of the plasma membrane, release of intracellular constituents, and induction of a strong inflammatory response (12). Necrosis often coexists with apoptosis and both types of cell death are observed simultaneously in many systems including tissue injury due to ischemia-reperfusion, toxic chemicals, and viral infections (13, 14). Although apoptosis of *Mtb*-infected Mϕ is associated with diminution of the infection, preponderance of necrosis has been associated with increased bacterial growth (3, 4).

There was some indication that the condition of the mitochondria is the branch point leading either to necrosis or to apoptosis. Therefore, we set up an experimental approach that favors either apoptosis or necrosis. Ca^{2+} is an intracellular messenger involved in cell death (15, 16). In HepG2 cells, increases in $[Ca^{2+}]_m$ lead to mitochondrial permeability transition (MPT) and to apoptosis (17). Therefore, we performed experiments which either favored apoptosis or necrosis by manipulation of the $[Ca^{2+}]_m$.

*Division of Rheumatology and Immunology, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, and [†]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

Received for publication July 9, 2002. Accepted for publication September 10, 2002.

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[1] This study was supported by National Institutes of Health Grants AI50216 and HL64884.

[2] L.D. and H.G. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Heinz G. Remold, Division of Rheumatology and Immunology, Department of Medicine, Brigham and Women's Hospital, Smith Building, Room 526B, 75 Francis Street, Boston, MA 02115. E-mail address: hremold@rics.bwh.harvard.edu

[4] Abbreviations used in this paper: *Mtb*, *M. tuberculosis*; Mϕ, macrophage; MPT, mitochondrial permeability transition; $\Delta\Psi_m$, mitochondrial membrane potential; RR, ruthenium red; $DiOC_6(3)$, 3,3′-dihexyloxycarbocyanine iodide; CHX, cycloheximide; PARP, poly(ADP-ribose) polymerase; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_m$, intramitochondrial Ca^{2+} concentration; PI, propidium iodide.

0022-1767/02/$02.00

Materials and Methods

Materials

Ca^{2+} ionophore A23187, cyclosporin A, ruthenium red (RR), propidium iodide (PI), and cycloheximide (CHX) were from Sigma-Aldrich (St. Louis, MO), and streptolysin O was from Cogenix (Peterborough, U.K.). 3,3′-dihexyloxycarbocyanine iodide ($DiOC_6(3)$), pluronic-F27, and Fluo-3 were from Molecular Probes (Eugene, OR). Murine monoclonal anti-caspase-3 Abs were purchased from Transduction Laboratories (Lexington, KY), and goat anti-caspase-9 Ab from Oncogene Research Products (San Diego, CA). Ab against cytochrome *c* were a kind gift of Dr. R. Jermmerson (University of Minnesota Medical School, Minneapolis, MN). Mouse anti-human poly(ADP-ribose) polymerase (PARP) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Bacteria

The attenuated *Mtb* strain H37Ra (American Type Culture Collection, Manassas, VA) was grown in 7H9 broth (Difco, Detroit, MI) with 10% BSA-glucose-catalase supplement (BD Biosciences, Mountain View, CA) and 0.05% Tween 80 (Difco) and resuspended in 7H9 broth at 5×10^7 CFU/ml.

Cells and culture

Mononuclear cells from peripheral blood of healthy donors after obtained informed consent were isolated as previously described (7). Mϕ for the in situ TUNEL assay were cultured on plastic cover slips (Nunc, Rochester, NY) and were plated at 2.5×10^6 mononuclear cells/ml/well in 24-well cluster plates (Costar, Cambridge, MA). Mϕ used for Western blot analysis were cultured on Costar 100-mm diameter tissue culture dishes (Costar) at 2×10^7 mononuclear cells/10 ml/dish. The resulting Mϕ population (2.5×10^5 cells/coverslip or 2.0×10^6 cell/dish) were 97–99% pure as determined by nonspecific esterase staining. The Mϕ were cultured in IMDM with 10% pooled human serum for 7 days to allow for Mϕ differentiation before *Mtb* infection. Mϕ were infected with five bacteria per cell.

Quantitation of mycobacteria

Counting of mycobacteria using the Bactec model 460TB system (BD Biosciences) was performed as described (7).

In situ analysis of programmed cell death

Apoptosis of adherent Mϕ was measured using a fluorescent in situ TUNEL assay (In Situ Cell Death Detection kit, tetramethylrhodamine-dUTP, catalog no. 2156792; Roche Biosciences, Indianapolis, IN) according to the specifications of the manufacturer. Mϕ necrosis was determined by PI staining of nuclei (18). In brief, following the experimental procedures, glass-adherent Mϕ were incubated with 10 μM PI at room temperature for 10 min. The cells were then washed two times with PBS, the coverslip dried, and PI-positive cells evaluated by fluorescence microscopy. Of note, PI stains at the early time points tested in these experiments only nuclei of cells whose membranes are permeable to this dye which is a sign for necrosis. The cell membranes of apoptotic Mϕ are impermeable to PI, unless the Mϕ undergo secondary necrosis. The assays were performed not later than 72 h after initiation of the cell culture, because at later time points cell membranes of apoptotic Mϕ become permeable to PI, which indicates the onset of secondary necrosis. In cultures harvested at and before 72 h, $<$10% of the total adherent cells present at time 0 are dislodged, which guarantees that only a minimum of total cells had been lost.

Cytochrome c *release from mitochondria*

Mϕ cultured in 24-well culture plates were washed twice with cold PBS and 1 ml of 1 μg/ml streptolysin O in PBS was added to permeabilize the cells and the plates were incubated at 4°C for 5 min. The streptolysin O solution was replaced with 1 ml of transport buffer (78 mM KCl, 4 mM $CaCl_2$, 50 mM HEPES buffer (pH 7.2), 2 mM DTT, and 1 μg/ml of protease inhibitors). After incubation at 37°C for 5 min and on ice for 20 min the cells were dislodged with a rubber policeman and pelleted at $500 \times g$. Supernatants were analyzed by Western blotting for cytochrome *c* released from the mitochondria. The cells were washed twice with PBS, dissolved in 1 ml of lysis buffer containing 250 mM NaCl, 50 mM HEPES buffer (pH 7.0), 0.1% Nonidet P-40, 50 mM NaF, 5 mM EDTA, 1 mM DTT, 0.3 mM PMSF, incubated on ice for 10 min and centrifuged at $10{,}000 \times g$ for 10 min. Protein concentrations were measured using the Bradford assay. Fifty micrograms of cell extract were processed to determine cytochrome *c* remaining in the mitochondria.

Determination of caspase activation

Mϕ were preincubated for 15 min and for the remaining time of the experiment with CHX (10 μg/ml) which blocks de novo protein synthesis to increase the sensitivity of the assay. Following the experimental procedures, Mϕ were treated at 4°C with lysis buffer for 1 h at 4×10^6 cells/ml and centrifuged at $15{,}000 \times g$ for 10 min. Fifty micrograms of cell lysates were heated in 2× sample buffer at 95°C for 5 min and resolved in 7.5 or 12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and blocked with 10 mM Tris buffer (pH 7.5) containing 150 mM NaCl, 0.05% Tween 20, and 5% dry milk for 2 h. The membranes were incubated with Ab (1 μg/ml) against caspase-3, caspase-9, cytochrome *c*, or PARP. Isotype-matched irrelevant Abs were used as controls. Membranes were then washed and blotted with HRP-protein A (Zymed Laboratories, San Francisco, CA). After extensive washing with 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 1% Tween 20, the membranes were developed in CL reagent (NEN, Boston, MA) and exposed to x-ray film.

Assessment of MPT induction in Mϕ (19)

MPT induction was assessed in Mϕ to evaluate mitochondrial membrane potential ($\Delta\Psi_m$) by measuring the retention of the lipophilic cationic dye $DiOC_6(3)$ within mitochondria as a consequence of the $\Delta\Psi_m$ (20). MPT results in the loss of $\Delta\Psi_m$ which causes decreased retention of $DiOC_6(3)$ in the mitochondria. Following the experimental procedures, CHX pretreated Mϕ cultured in 6-well plates were washed twice with PBS. The cells were then incubated for 30 min with 1 nM $DiOC_6(3)$ at 37°C. Digitonin was then added to a final concentration of 7.5 μM for 20 min, the cells were washed three times with PBS, fixed with 1% formaldehyde for 20 min, and washed twice with PBS. Thereafter, the cells were dislodged from the plates using a rubber policeman and analyzed for fluorochrome incorporation in a BD FACSort flow cytometer (BD Biosciences). The Mϕ population with reduced $DiOC_6(3)$ was determined by setting the gate to allow separation of the cell population with reduced staining from the Mϕ population with intact mitochondria showing high staining with $DiOC_6$7(3). Percentage of Mϕ with reduced $DiOC_6(3)$ staining of the total gated cell population was determined.

Electron microscopy

Mϕ were cultured in IMEM containing 10% human serum in 10-cm plastic petri dishes. Following the experimental procedures, the cells were washed three times in 0.1 M sodium cacodylate (pH 7.3) and fixed with 5 ml of MJK/2 solution for 2 h, removed by using a rubber policeman, transferred to microcentrifuge tubes, and stored overnight at 4°C. The cells were then exposed to 1% uranyl acetate in water for 15 min at room temperature, washed twice with distilled water, centrifuged into 3% agarose at 45°C, and cooled to form blocks. The agarose blocks were dehydrated using graded steps of acetone and embedded in Spurr's low viscosity media. After polymerization at 65°C overnight, 80-nm sections were cut and picked up with copper grids which were then stained with uranyl acetate and bismuth subnitrate. The sections were analyzed with a Jeol electron microscope (Jeol USA, Peabody, MA) and recorded on Kodak sheet film (Eastman Kodak, Rochester, NY).

Determination of Ca^{2+} flux

Mϕ were plated on glass coverslips in 24-well plates, 1.0×10^5 cells/well, in calcium-free IMEM without phenol red containing 10% pooled human serum. After the cells were inoculated with *Mtb* in absence or presence of A23187 from 15 min to 2 h, the cells were loaded with 0.5 ml fluo-3/AM solution (10 ml HEPES buffer (pH 7.2) containing 60 μg Fluo-3/AM, 10 μl Pluronic F-127, and 40 μl of 1 M probenecid). The cells were then incubated for 30 min at room temperature and washed three times with PBS. Following experimental procedures, the cells were washed and the coverslips containing the Mϕ transferred into Mackaness-type chambers. The chambers were filled with IMEM containing 10% human serum. Accidental infection with *Mtb* was prevented by containment of the bacteria within the Mackaness-type chambers. Ca^{2+} flux was assessed by mounting the Mackaness-type chamber on a Biophysica microscope chamber which had been modified for use with an ACAS 570 Ultima Interactive Laser Cytometer (Meridian Instruments, Okemos, MI; Ref. 21). Changes in $[Ca^{2+}]_i$ were assessed by fluorescence analysis of Mϕ at an excitation wavelength of 488 nm and an emission wavelength of $>$515 nm, quantified as relative fluorescence intensity and displayed on a pseudocolor intensity scale.

Statistical analysis

Results are expressed as mean ± SEM. The data were analyzed using SigmaStat Statistical Software (Jandel, San Rafael, CA) using a *t* test for normally distributed data with equal variances and the Mann-Whitney *U* rank sum test for data populations with nonnormal distributions and/or unequal variances.

Results

Infection of Mϕ with Mtb *induces both apoptosis and necrosis*

Mϕ cultures incubated with *Mtb* for 0, 24, 48, and 72 h were assayed for apoptotic and necrotic cells. Apoptic adherent Mϕ were determined by fluorescent microscopy using a TUNEL assay and necrotic Mϕ by PI staining of the nuclei (see *Materials and Methods*). At all indicated time points, <5% of the cultured Mϕ became detached and were lost. At 48 and 72 h, ~50% of Mϕ were apoptotic and 15–50% of the Mϕ were necrotic (Fig. 1), demonstrating that inoculation with *Mtb* induces cell death with the characteristics of both apoptosis and necrosis (14, 15). Inoculation of Mϕ with *Mtb* resulted also in moderate loss of $\Delta\Psi_m$ as manifested by decreased mitochondrial accumulation of $DiOC_6(3)$, by release of cytochrome *c* from the mitochondria, and activation of the caspases-9 and -3. (see Figs. 5, 6, and 8).

The Ca^{2+} ionophore A23187 blocks necrosis, promotes apoptosis, and enhances antimycobacterial activity of the Mϕ

We next investigated whether inoculation of Mϕ with *Mtb* has an effect on calcium flux. Inoculation with *Mtb* moderately increased calcium flux into the Mϕ which was detectable at 2 h (data not shown) and maximal at 4 h (Fig. 2, *upper right panel*). Addition of A23187 further increased calcium levels (Fig. 2, *lower left panel*) and the L-type Ca^{2+} channel blocker dilthiazem completely inhibited *Mtb*-induced calcium flux (Fig. 2, *lower right panel*).

To investigate whether increase of $[Ca^{2+}]_i$ blocks necrosis, Mϕ were infected in the presence of 0.5 μM A23187. With A23187, the percentage of apoptotic Mϕ at 48 h was similar to that without A23187 (44 ± 1% vs 46 ± 2% in Mϕ infected without A2318, $n = 3$), while a significant decrease of necrotic cells was seen in comparison to Mϕ cultures infected without A23187 (Fig. 3). Lower concentrations of A23187 had no effect (data not shown). Less than 5% necrotic Mϕ were seen in noninfected cultures with or without A23187 (data not shown). Although it is well recognized that $[Ca^{2+}]_i$ overload caused by ionophore leads to cell death (22, 23), the increase of $[Ca^{2+}]_i$ induced by A23187 alone did not induce Mϕ mortality (data not shown).

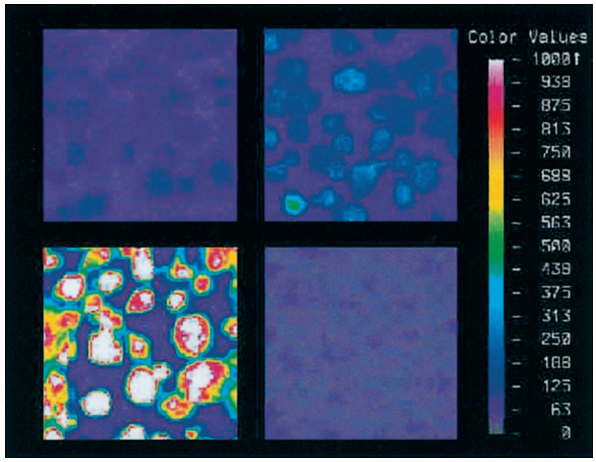

FIGURE 2. Ca^{2+} flux in Mϕ untreated (*upper left panel*), infected with *Mtb* (5 organisms/cell) (*upper left panel*), infected with *Mtb* and treated with 0.5 μM A23187 (*lower left panel*), and Mϕ infected and treated with 2 μM dilthiazem (*lower right panel*). Ca^{2+} flux was determined 4 h after infection. Relative $[Ca^{2+}]_i$ in single cells is indicated on a pseudocolor scale of fluorescence intensity by the colors indicated on the *right*. The picture images are taken from a typical experiment of three experiments.

To determine whether block of necrosis correlates with inhibition of mycobacterial growth, we induced predominantly apoptosis by infecting Mϕ in presence of A23187 and measured the number of bacteria in the cultures. *Mtb* replication in Mϕ cultures infected with *Mtb* was significantly restricted after 48 h in presence of increased concentrations of A23187 (Fig. 4, *right panel*; Ref. 24) in comparison to *Mtb* growth in Mϕ cultured without additives paralleling the marked abrogation of Mϕ necrosis in presence of A23187 (Fig. 4, *left panel*).

A23187 prevents cytochrome c *release from Mϕ mitochondria and diminishes caspase activation in* Mtb*-infected Mϕ*

Release of cytochrome *c* from the mitochondria is a critical requirement for induction of programmed cell death (8, 9). Therefore, we determined whether release of cytochrome *c* from mitochondria is altered by A23187. In Mϕ infected with *Mtb*, ~40% of

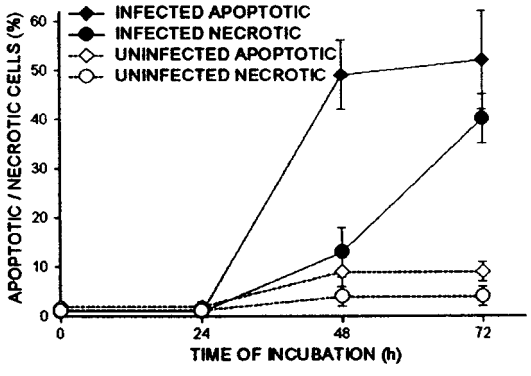

FIGURE 1. Time course of apoptosis and of necrosis of Mϕ infected with *Mtb* (5 organisms/cell). Infected and uninfected Mϕ were tested for apoptosis (◇, ◆) and for necrosis (○, ●) at the indicated time points. The difference in percentage of apoptosis between Mϕ inoculated with *Mtb* and uninfected Mϕ is statistically significant at 48 h ($p = 0.0027$, $n = 3$) and 72 h ($p = 0.015$, $n = 3$). The difference in percentage of necrosis between infected and uninfected Mϕ cultures among the treatments at 48 h ($p = 0.06$, $n = 3$ and *Mtb* vs *Mtb* + RR, $p = 0.03$, $n = 3$) and at 72 h ($p = 0.004$, $n = 3$) is statistically significant.

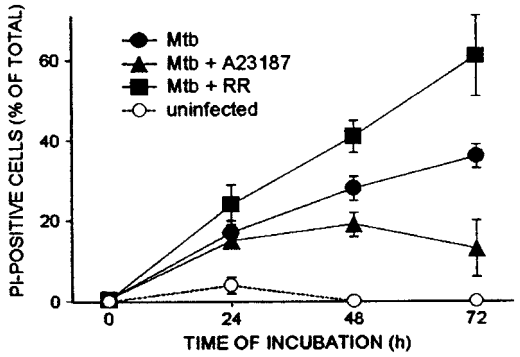

FIGURE 3. Time course of necrosis of Mϕ infected with *Mtb* in presence of A23187 or RR. Mϕ remained uninfected (○) or were infected in absence (●), in presence of 0.5 μ M A23187 (▲), or in presence of 5 μg/ml RR (■). The differences in percentage of necrosis among the treatments at 48 h (*Mtb* vs *Mtb* + A23187, $p = 0.06$, $n = 3$, and *Mtb* vs *Mtb* + RR, $p = 0.03$, $n = 3$) and at 72 h (*Mtb* vs *Mtb* + A23187, $p = 0.004$, $n = 3$, and *Mtb* vs *Mtb* + RR, $p = 0.006$, $n = 3$) are statistically significant.

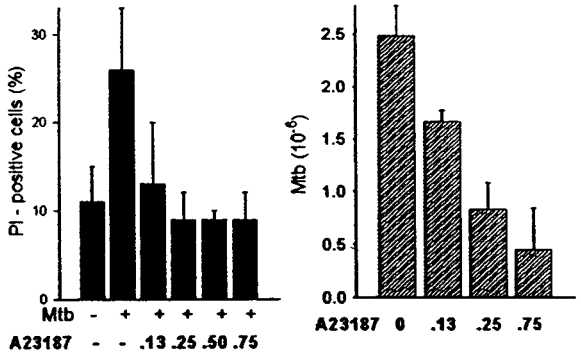

FIGURE 4. Effect of increasing concentrations of A23187 on necrosis of Mϕ infected with *Mtb* (*left panel*) and on mycobacterial growth (*right panel*) in Mϕ cultures. Mϕ were infected with 5 *Mtb*/Mϕ for 48 h in the presence of the indicated concentrations of A23187. Note that *Mtb* growth in Mϕ cultures was significantly restricted after 48 h with 0.13–0.75 μM A23187 (Fig. 3, *right panel*) compared with cultures without additives ($p = 0.04$, $n = 4$), paralleling the marked abrogation of Mϕ necrosis (Fig. 3, *left panel*, $p = 0.005$, $n = 3$).

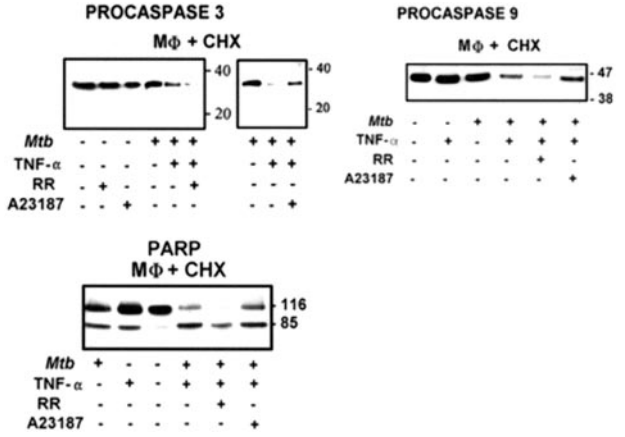

FIGURE 6. Caspase-3 and caspase-9 activation and PARP degradation in *Mtb*-infected Mϕ is inhibited by A23187 and up-regulated by RR. CHX-treated Mϕ were infected or not infected with *Mtb* in presence or absence of A23187 (0.5 μM) or RR (5 μg/ml). After 24 h, the cells were harvested and examined by Western blotting for procaspase-3 (32 kDa) and procaspase-9 (47 kDa) degradation and PARP (116 kDa) degradation into the main proteolytic product of PARP (85 kDa). Anti-actin Ab was used in parallel as a loading control (data not shown).

total cytochrome *c* was released from the mitochondria (Fig. 5, *first lane from right*), indicating that *Mtb* infection increases permeability of the inner mitochondrial membrane to cytochrome *c*. Increasing $[Ca^{2+}]_i$ diminished mitochondrial release of cytochrome *c* significantly (Fig. 5, *fourth lane from left*).

To determine whether activation of caspase-3 and caspase-9 correlates with necrosis or apoptosis, we assessed the activation of caspase-3 and caspase-9 in *Mtb*-infected Mϕ in presence of A23187 by determining the amount of residual procaspase. In these experiments, Mϕ were preincubated with CHX to prevent synthesis of procaspases during culture. In CHX-pretreated Mϕ, both *Mtb* and TNF-α needed to be added to induce activation of caspase-3 and caspase-9 and degradation of PARP, an important caspase-3 substrate (Ref. 25; 70 and 60% loss of procaspase-3 and procaspase-9 and 45% PARP degradation, respectively, Fig. 6). A23187 decreased procaspase-3 activation and procaspase-9 activation (29 and 15% loss of procaspase-9 and procaspase-3, respectively), although the percentage of apoptotic Mϕ in the cultures was increased (Fig. 1). The inhibitory effect of A23187 on degradation of PARP was minimal.

The Ca^{2+} uniporter inhibitor RR enhances necrosis of Mtb*-infected Mϕ and mycobacterial replication in* Mtb*-infected Mϕ cultures*

RR, an inhibitor of the mitochondrial Ca^{2+} uniporter, blocks Ca^{2+} flux through mitochondrial membranes and decreases $[Ca^{2+}]_m$ (26–28). In presence of 5 ng/ml RR, a marked increase in percentage of necrotic Mϕ after inoculation with *Mtb* was seen at 48 and 72 h compared with Mϕ cultures infected without RR (Fig. 3). In contrast, the percentage of apoptotic Mϕ was not different from that in Mϕ cultures infected without RR (47 $\pm$ 9% vs 46 $\pm$ 2, $n = 3$). Uninfected Mϕ cultures incubated with A23187 (data not shown), or with RR alone (data not shown) did not show an increase of necrosis over background levels. Mϕ necrosis augmented by RR (Fig. 7, *left panel*) correlated with an increase of mycobacterial replication (Fig. 7, *right panel*).

We confirmed these findings using the L-type Ca^{2+} channel inhibitor dilthiazem known to decrease $[Ca^{2+}]_i$ (29) leading to decreased $[Ca^{2+}]_m$. Addition of 2 μM dilthiazem to infected Mϕ increased the amount of necrotic Mϕ after 3 days from 37 $\pm$ 1 to 53 $\pm$ 2% ($p = 0.0001$, $n = 3$). No increase of necrosis was observed in the presence of dilthiazem alone (data not shown). These results indicate that decrease of $[Ca^{2+}]_i$ induces predominantly necrosis after infection with *Mtb*, but does not induce Mϕ death in absence of infection.

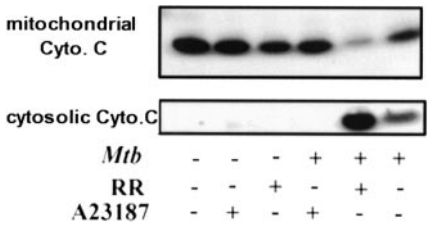

FIGURE 5. Mitochondrial cytochrome *c* release of Mϕ infected with *Mtb* is inhibited by A23187 and enhanced by RR. Mϕ were infected for 48 h with 5 *Mtb*/cell in absence and presence of 5 μg/ml RR or 0.5 μM A23187. Cytochrome *c* present in the mitochondria and in the cytosol was determined by Western blotting. Anti-actin Ab was used as a loading control (data not shown).

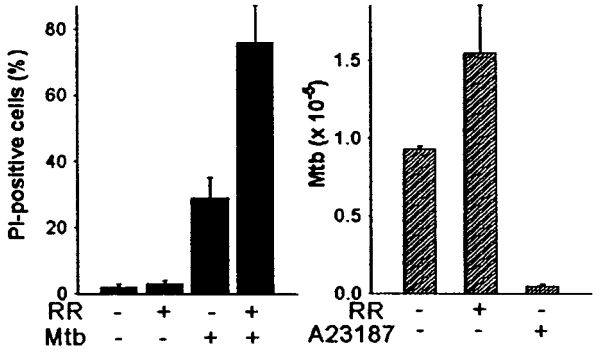

FIGURE 7. Effect of RR in *Mtb*-infected Mϕ cultures on necrosis (*left panel*) and on mycobacterial replication (*right panel*). Mϕ were infected for 48 h without or with RR. Necrosis (*left panel*, $p = 0.02$, $n = 4$) and mycobacterial replication (*right panel*, $p = 0.05$, $n = 3$) were significantly increased. In contrast, addition of A23187 significantly blocked mycobacterial replication.

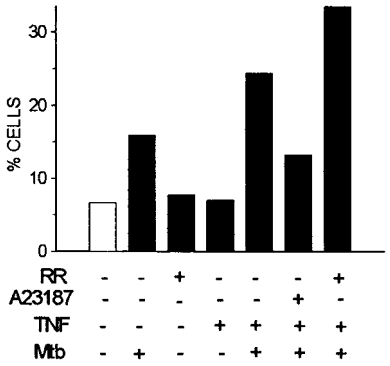

FIGURE 8. MPT in Mϕ infected with *Mtb* is increased by RR and blocked by A23187. CHX and $DiOC_6(3)$-preincubated Mϕ were infected for 48 h with 5 *Mtb*/cell without and with TNF-α, 5 μg/ml RR, and of 0.5 μ M A23187. The cell population with reduced dye retention was gated. Cationic dye retention in mitochondria of Mϕ infected with *Mtb* in presence of RR was statistically significantly lower than dye retention in mitochondria of Mϕ incubated with *Mtb* and TNF-α and with *Mtb*, TNF-α, and A21387 ($p = 0.001$, $n = 3$).

RR increases cytochrome c *release from Mϕ mitochondria and enhances caspase activation*

In presence of RR, *Mtb* inoculation dramatically enhanced cytochrome *c* release from the mitochondria resulting in 75% release of total cytochrome *c* (Fig. 5, *second lane from right*). Addition of RR to uninfected Mϕ had no effect (Fig. 5, *third lane from left*).

Furthermore, RR strongly enhanced caspase-9 activation, caspase-3 activation, and PARP degradation in CHX-pretreated Mϕ infected for 12 h with *Mtb* in presence of TNF-α (90, 80, and 94% decrease of procaspase-9, procaspase-3, and PARP, respectively, compared with Mϕ infected without RR; Fig. 6). RR alone had no effect on caspase activation and PARP degradation (Fig. 6, *upper left panel*, data not shown for caspase-9 and PARP). It should be noted that necrosis of RR-treated cells is not caused by increased activation of PARP leading to ATP depletion (30), because addition of RR also markedly enhanced degradation of PARP (Fig. 6).

Infection of Mϕ in presence of RR causes irreversible MPT pore opening and mitochondrial swelling

We further investigated whether MPT is required for induction of necrosis or apoptosis. MPT causes irreversible breakdown of the mitochondrial transmembrane potential $\Delta\Psi_m$ (31) which correlates with the release of cytochrome *c* from mitochondria. To assess MPT, accumulation of the fluorescent cationic dye $DiOC_6(3)$ (20) in the mitochondrial matrix was determined as a consequence of the $\Delta\Psi_m$. Release of $DiOC_6(3)$ from the mitochondria is considered a reliable indicator for $\Delta\Psi_m$ loss and of MPT pore opening (20).

Optimal results were obtained when we measured the retention of $DiOC_6(3)$ within mitochondria in Mϕ pretreated with CHX at day 3. A significant decrease of dye retention in the mitochondria was seen in Mϕ infected with *Mtb* alone as compared with noninfected controls which was enhanced by addition of TNF-α. A substantial loss of dye retention was measured in infected cultures incubated with TNF-α and RR, indicating significant MPT (Fig. 8, *lane 1 from the right*). Mϕ incubated with *Mtb*, TNF-α, and A23187 alone showed dye retention similar to cell cultures infected with *Mtb* in absence of TNF-α (Fig. 8, *lane 4 from right*). These experiments clearly demonstrate that massive mitochondrial damage is caused by *Mtb* in presence of RR that leads to loss of $DiOC_6(3)$ retention as the consequence of MPT and irreversible

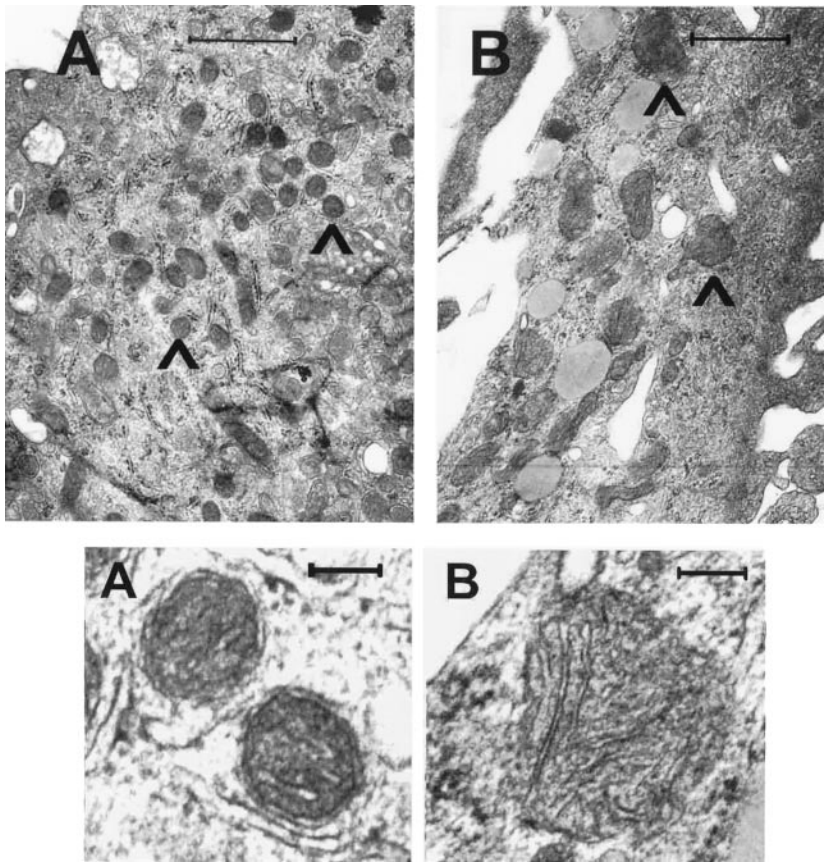

FIGURE 9. *Mtb* induces mitochondrial swelling of Mϕ incubated with RR. RR-treated Mϕ remained uninfected (*A*) or were infected with *Mtb* (*B*) for 24 h and examined by transmission electron microscopy for mitochondrial swelling. *Upper panels*, Low power electron micrographs of Mϕ (the bar represents 1 μm); *lower panels*, higher power electron micrographs of single mitochondria (the bar represents 0.1 μm). Note that Mϕ incubated with RR and *Mtb* show swelling of the mitochondria and loss of the outer mitochondrial membrane (*B*, *lower panel*). Mitochondria of Mϕ incubated without additive or infected with *Mtb* in absence of RR showed identical appearance to those in *A* (data not shown).

$\Delta\Psi_m$ loss. A23187 prevents mitochondrial damage and loss of dye retention.

Similar results were obtained in electron microscopic studies that assessed mitochondrial swelling. Mϕ incubated with *Mtb* and RR for 4 days show significant mitochondrial swelling indicating mitochondrial membrane damage, whereas uninfected Mϕ and Mϕ treated with RR alone contained normally sized mitochondria (Fig. 9).

Discussion

Mϕ inoculated with *Mtb* undergo apoptosis, which is associated with strong antimycobacterial activity. Experiments with virulent mycobacteria suggested that these pathogens are able to override the detrimental effects of apoptosis by inducing necrosis, which results in uncontrolled mycobacterial growth (4, 5). We hypothesized that the branch point for either apoptosis or necrosis is the mitochondrial membrane permeability and that the mitochondrial membrane permeability depends on $[Ca^{2+}]_i$.

Ca^{2+} is known to be a universal secondary messenger indispensable in the majority of cellular signal transduction pathways including fertilization, proliferation, and development. Ca^{2+} signaling is important for membrane excitability, mitochondrial metabolism, vesicle secretion, mitosis, muscle contraction, NO production, and in the induction of cell death (32).

Previously, increase of $[Ca^{2+}]_i$ in Mϕ was found to maintain mitochondrial integrity (17, 33) and to cause apoptosis (15–17). The present study demonstrates that increase of intracellular Ca^{2+} protects mitochondria from irreversible damage by *Mtb*, promotes apoptosis, and inhibits Mϕ necrosis and mycobacterial survival.

However, intracellular Ca^{2+} concentrations seem to have a broad effect on multiple antimycobacterial mechanisms including defense mechanisms that are not dependent on mitochondrial function. Block of calcium influx was found to have a direct inhibitory effect on phagosome-lysosome fusion (24, 34). Down-regulation of calcium influx resulting in block of Mϕ maturation clearly correlates with a significantly decreased defense capacity against mycobacteria in Mϕ which do not undergo programmed cell death (24). The importance of this finding is underscored by the observation that virulent *Mtb* are able to inhibit Ca^{2+} signaling and Mϕ phagosome-lysosome fusion which results in increased intracellular survival of the *Mtb* (24). Therefore, the action of intracellular calcium resulting in antimycobacterial defense is not always dependent on the status of the Mϕ with respect to programmed cell death.

It was suggested that moderate increase of $[Ca^{2+}]_i$ up-regulates the cell's energy state and thereby protects mitochondrial integrity (33, 35). Consequently, ATP depletion (36) was found to lead to irreversible loss of $\Delta\Psi_m$ and to necrosis (37). In contrast, selective reconstitution of the extramitochondrial ATP pool with glucose prevented necrosis in ATP-depleted cells and restored the ability of the cells to undergo apoptosis (38). In Mϕ, ATP is also necessary for increased antimycobacterial activity (39).

Our studies further indicate that increased caspase activation leads to Mϕ necrosis, but the possibility that increased caspase activation under necrotic conditions is the result of a general breakdown of cellular functions cannot be excluded.

The effect of Ca^{2+} depletion on the mitochondria themselves is of critical importance leading to disruption of the mitochondrial inner membrane potential and to necrosis of the Mϕ (40). Our findings support the hypothesis of Kroemer et al. (41) that the relative quantity of apoptogenic factors released vs the extent of mitochondrial dysfunction is responsible for induction of either apoptosis or necrosis. In intact mitochondria, the transmembrane potential ($\Delta\Psi_m$) keeps the inner portion of the inner mitochondrial membrane negatively charged, which allows accumulation of the cationic dye $DiOC_6(3)$ in the mitochondrial matrix. Reduction of $DiOC_6(3)$ retention within the mitochondria is an indicator for loss of $\Delta\Psi_m$, which is the consequence of MPT. Necrosis of *Mtb*-infected Mϕ in presence of RR correlates with reduced $DiOC_6(3)$ retention within the mitochondria and indicates the collapse of $\Delta\Psi_m$. High $[Ca^{2+}]_i$ induced by A23187 rescues mitochondria from irreversible damage which manifests itself in an increase of $DiOC_6(3)$ retention within the mitochondria and Mϕ apoptosis. In agreement with this model, HepG2 cells exposed to proapoptotic stimuli seem to undergo reversible mitochondrial membrane pore opening. Reversible mitochondrial pore opening was postulated on the basis of a transient increase in $[Ca^{2+}]_i$ that caused a pulse of cytochrome *c* release from the mitochondria and apoptosis. Apoptosis was thought to be dependent on resealing of the pores and prompt recovery of the mitochondrial energy metabolism. Despite ongoing caspase activation, the mitochondrial metabolism required for apoptosis was found to be intact (17, 22).

We hypothesize that a similar mechanism is effective in *Mtb*-infected Mϕ in which A23187 induces a transient increase of $[Ca^{2+}]_i$. The ensuing increase of $[Ca^{2+}]_m$ (23) is thought to be due to the presence of a passive uniporter in the mitochondrial membrane, a structure that increases ion diffusion down the electrochemical gradient and does not couple the transport to that of any other ion (42). In presence of A23187, no reduction of $DiOC_6(3)$ retention in the mitochondria was observed, suggesting that in presence of high $[Ca^{2+}]_m$, mitochondrial pore opening is reversible and does not lead to permanent breakdown of $\Delta\Psi_m$, resulting in apoptosis rather than in necrosis.

Although in infected Mϕ, A23187 down-regulates cytochrome *c* release, caspase activation and apoptosis were clearly detectable. We speculate that our method used to detect cytochrome *c* release from the mitochondria, Western blotting, is not sensitive enough to measure the small amounts of cytochrome *c* released in the presence of A23187 that are nevertheless sufficient for activation of procaspase-9 and for triggering of the apoptotic cascade. Alternatively, unrelated mechanisms may lead to caspase-9 activation (43, 44).

The suggested transient nature of mitochondrial pore opening further implies that release of cytochrome *c* into the cytosol does not require collapse of $\Delta\Psi_m$, an event that leads to irreversible MPT pore opening (45–47) combined with wholesale release of apoptogenic mediators (48). Several studies indicating that cytochrome *c* is released from mitochondria early in the onset of apoptosis in the absence of MPT are in line with our studies (49–52) and suggest that irreversible MPT is not required for cytochrome *c* release. Therefore, we propose that a delicate balance between signals is required in infected Mϕ to induce apoptosis and *Mtb* elimination, because on one hand sufficient amounts of apoptogenic factors need to be released from the mitochondria, but in contrast mitochondria need to remain intact to prevent necrosis. Our studies strongly indicate a key role for calcium in the outcome of the type of cell death.

Published evidence indicates that inhibition of Ca^{2+} transport into the mitochondria by RR leads to irreversible decrease of $[Ca^{2+}]_m$ (28). We could document loss of $\Delta\Psi_m$, mitochondrial swelling, enhanced necrosis, and bacterial growth in Mϕ incubated with *Mtb* and RR as a possible consequence of $[Ca^{2+}]_m$ breakdown.

Surprisingly, both drugs, A23187 and RR, have no or only a slight effect on the survival of uninfected Mϕ, indicating that signals emanating from the mycobacteria are the primary inducers of cell death.

Cumulatively, these findings lead to the conclusion that high $[Ca^{2+}]_i$ required for effective maintenance of mitochondrial functions after infection of the Mϕ with *Mtb* is of critical importance for the defense against *Mtb*. Therefore, we hypothesize that drugs which increase $[Ca^{2+}]_i$ and aid in the protection of mitochondrial integrity might be suitable therapeutic tools to bolster innate immune defense mechanisms against *Mtb*.

Acknowledgments

We thank Patrick Yacono (Harvard Medical School, Boston, MA) for the help in performing the calcium measurements.

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