Enhancement of Antimycobacterial Activity of Macrophages by Stabilization of Inner Mitochondrial Membrane Potential

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Infection of human macrophages with Mycobacterium tuberculosis leads to cell death that, depending on the M. tuberculosis strain, time course, and multiplicity of infection, may have predominant features of apoptosis or necrosis. A key feature of infection-induced necrosis is mitochondrial damage characterized by an irreversible increase in the mitochondrial permeability transition (MPT), which is associated with increased release of cytochrome c from the mitochondria and uncontrolled mycobacterial replication. In contrast, protection of the mitochondria from MPT favors apoptosis of M. tuberculosis–infected macrophages. Apoptosis of M. tuberculosis–infected macrophages is associated with killing of intracellular M. tuberculosis, and this may be enhanced when MPT is stabilized. Here, we show that cyclosporin A (CsA), an inhibitor of MPT, protects the mitochondria from release of cytochrome c and promotes the antimycobacterial activity of macrophages infected with M. tuberculosis H37Ra. Signal by purinergic P2 receptors has previously been linked to the antimycobacterial activity of macrophages. In the present study, we found that infection with H37Ra inhibits P2X, receptor (P2XR) signals and that CsA restores P2XR function in infected macrophages. Together, these data demonstrate that CsA promotes at least 2 antimycobacterial pathways of macrophages.

The alveolar macrophage is the first line of defense against Mycobacterium tuberculosis, but the bacillus has the capacity to survive in macrophages and to use these cells as a growth niche to establish infection in the host [1]. Human macrophages may respond to M. tuberculosis infection by undergoing tumor necrosis factor (TNF)–γ–mediated apoptosis [2]. We have previously found that, at low MOIs, attenuated M. tuberculosis H37Ra or M. bovis bacille Calmette-Guérin induces more apoptosis in macrophage cultures than do virulent strains, such as H37Rv [3]. Apoptosis of infected macrophages is associated with killing of intracellular mycobacteria and might also benefit the host by sequestering bacilli in apoptotic bodies for subsequent expedited uptake by phagocytes [3–6]. This would prevent the extracellular spread of infection and protect surrounding tissues from the harmful effects of cellular necrosis. Macrophages infected with mycobacteria may also undergo death that resembles necrosis, which leads to rampant mycobacterial growth [5]. We implicated mitochondrial damage, on the basis of the irreversible opening of the mitochondrial permeability transition (MPT) pore, as a pivotal mechanism linked to necrosis of infected macrophages [7]. Important characteristics of the irreversible opening of the MPT pore include loss of inner mitochondrial membrane potential, increased release of cytochrome c into the cytosol, pronounced mitochondrial swelling, enhanced activation of caspase, and less-effective control of mycobacterial growth. In contrast, when MPT is blocked, irreversible damage of the mitochondrial membrane is inhibited and antimycobacterial mechanisms in apoptotic cells remain in effect.
On the basis of these findings, we became interested in establishing conditions that attenuate MPT after infection with *M. tuberculosis*. We hypothesized that such conditions support the defense mechanisms of macrophages against mycobacteria. Cyclophilin D (Cyp-D), a component of the MPT pore that is located within the inner mitochondrial membrane, specifically binds cyclosporin A (CsA) [8, 9], leading to inhibition of MPT [10, 11]. The present study demonstrates that inhibition of MPT by CsA significantly enhances the antimycobacterial activity of macrophages against H37Ra.

The exact function of intact mitochondrial membrane integrity during mycobacteria-induced death of macrophages, in the defense against mycobacteria, is not understood. Several studies suggest that the function of purinergic P2 receptors (P2Rs), rather than the production of reactive oxygen or nitrogen intermediates, is of critical importance in the defense of macrophages against mycobacteria [12, 13]. Here, we demonstrate that infection of macrophages with H37Ra significantly inhibits the functional activity of P2R and that this activity is completely restored by CsA. These results suggest that CsA maintains the function of purinergic P2Rs, which is down-regulated by infection with mycobacteria. Whether this activity of CsA depends on mitochondrial membrane integrity is not known.

**MATERIALS AND METHODS**

**Materials.** CsA, ruthenium red, ethidium, and propidium iodide (PI) were obtained from Sigma, and streptolysin O was obtained from Cogenix. DiOC₆(3) was obtained from Molecular Probes. Murine monoclonal anti–caspase-3 antibody was obtained from Transduction Laboratories, goat anti–caspase-9 antibody was obtained from Oncogene, and antibody against cytochrome c was a gift from Ronald Jemmerson (University of Minnesota Medical School, Minneapolis).

**Bacteria.** The attenuated *M. tuberculosis* stain H37Ra and the virulent strain H37Rv (American Type Culture Collection) were grown in 7H9 broth (Difco) enriched with 10% bovine serum albumin–glucose-catalase supplement (BD Biosciences) and 0.05% Tween 80 (Difco) and were resuspended in 7H9 broth, at \(5 \times 10^7\) cfu/mL. To prevent formation of clumps, the bacteria were suspended in RPMI 1640 medium with 10% pooled human serum and were sonicated. After being allowed to settle for 10 min, the supernatants were transferred to a new tube and were allowed to settle for another 10 min. The supernatants were then checked for clumps by microscopy. Less than 10% of the bacteria were clumped. The concentration of viable bacteria was determined by Bactec counting.

**Quantification of mycobacteria.** Adherent macrophages were cultured with H37Ra or H37Rv, at an MOI of 1:1 or 5:1. After 4 h, the monolayers were washed 5 times with Hank’s balanced salt solution (HBSS) and cultured in Iscove’s modified Dulbecco’s medium (IMDM). To measure mycobacterial growth in the macrophage cultures, after infection, monolayers were cultured for various periods. The cells were lysed by addition of 500 \(\mu\)L of 0.2% SDS in PBS, and the effect of SDS was neutralized by addition of 500 \(\mu\)L of 50% fetal calf serum (FCS). Cell lysates (100 \(\mu\)L) from triplicate cultures were serially diluted 10-fold and plated on 7H10 agar plates (Remel). Colonies were counted after the plates had been incubated for 21 days at 37°C. Alternatively, the cell lysates were pooled and inoculated into triplicate Bactec 12B vials. The number of mycobacteria was then determined by use of the Bactec model 460TB system (BD Biosciences).

**Cells and culture.** Alveolar macrophages were obtained from bronchoalveolar lavage (BAL) fluid from healthy, non-smoking volunteers by use of standard techniques. Informed consent was obtained from the volunteers, and the guidelines for human experimentation of the US Department of Health and Human Services, Brigham and Women’s Hospital (Boston, Massachusetts), and the University of Massachusetts Medical Center (Worcester) were followed in the conduct of this research. BAL fluid was filtered through sterile gauze and centrifuged at 450 \(g\) for 10 min, and the cell pellet was then resuspended in RPMI 1640 medium (Life Technologies) with 10% FCS and 50 \(\mu\)g/mL cefotaxime. After incubation for 24 h, nonadherent cells were removed by washing. Differential cell counts were performed on cytocentrifuged preparations by use of the Leuko Stat Stain Kit (Fisher).

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated as described elsewhere [14]. Macrophages for the in situ TUNEL assay were cultured on plastic cover slips (Nunc) and were plated at \(10^6\) PBMCs/mL/well in 24-well cluster plates (Costar). Macrophages used for Western blot analysis were cultured on Costar 100-mm diameter tissue culture plates (Costar), at \(2.0 \times 10^7\) PBMCs/10 mL/plate. The resulting macrophage population (\(1.0 \times 10^6\) cells/cover slip or \(2.0 \times 10^5\) cell/plate) were 97%–99% pure, as determined by nonspecific esterase staining. Macrophages were cultured in IMDM with 10% pooled human serum for 7 days, to allow for differentiation of macrophages before *M. tuberculosis* infection. Macrophages were infected with either 1 bacterium/cell or 5 bacteria/cell for 4 h and then were washed 4 times with HBSS before further treatment. At 4 h, at least 60% \(\pm\) 10% (mean \(\pm\) SE) of the adherent macrophages were infected with bacteria.

**In situ analysis of programmed cell death.** Apoptosis of adherent macrophages was measured by use of a fluorescent in situ TUNEL assay (In Situ Cell Death Detection Kit; TMR Red; Roche), in accordance with the manufacturer’s specifications. Necrosis of macrophages was determined by PI or 7-amino actinomycin D staining of nuclei [15]. In brief, glass-adherent macrophages were incubated with 10 \(\mu\)g/mL PI for 10 min at room temperature and washed twice with PBS, the cover slip was dried, and PI-positive cells were evaluated by...
fluorescence microscopy. Alternatively, the cells were stained with 10 μg/mL 7-amino actinomycin D for 30 min at 4°C. After being washed twice with PBS, the cells were fixed in 1% paraformaldehyde containing 50 μg/mL actinomycin D. The cells were then washed and subjected to fluorescence-activated cell sorter (FACS) analysis. At the early time points, PI and 7-amino actinomycin D stained only nuclei of cells whose membranes were dye permeable, which is a sign of necrosis. The cell membranes of apoptotic macrophages are impermeable to PI and 7-amino actinomycin D but become permeable when the macrophages undergo secondary necrosis. In macrophage cultures harvested before 72 h, <10% of the total adherent cells present at time 0 were dislodged, which guaranteed that only a minimum of total cells had been lost to analysis. This number is higher in alveolar macrophage cultures.

Release of cytochrome c from the mitochondria. Macrophages cultured in 24-well culture plates were washed twice with cold PBS; 1 mL of 1 μg/mL streptolysin O in PBS was added to permeabilize the cells, and the plates were incubated for 5 min at 4°C. The streptolysin O solution was replaced with 1 mL of transport buffer (78 mmol/L KCl, 4 mmol/L CaCl₂, 50 mmol/L HEPES buffer [pH 7.2], 2 mmol/L dithiothreitol [DTT], and 1 μg/mL protease inhibitors). After incubation at 37°C (5 min) and on ice (20 min), the cells were dislodged by use of a rubber policeman and removed by centrifugation at 500 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 mmol/L NaCl, 50 mmol/L HEPES buffer (pH 7.0), 0.1% NP40, 50 mmol/L NaF, 5 mmol/L EDTA, 1 mmol/L DTT, and 0.3 mmol/L phenylmethylsulfonyl fluoride; incubated for 10 min on ice; and centrifuged at 10,000 g for 10 min. Protein concentrations were measured by use of the Bradford assay. Fifty micrograms of cell extract was processed to determine the amount of cytochrome c remaining in the mitochondria.

Determination of caspase activation. Macrophages were preincubated for 15 min and were incubated for the remaining time of the experiment with cycloheximide (CHX; 10 μg/mL), which inhibits de novo protein synthesis, to increase the sensitivity of the assay. After performance of the experimental procedures, macrophages were treated for 1 h at 4°C with lysis buffer, at a concentration of 4 × 10⁶ cells/mL, and centrifuged at 15,000 g for 10 min. Fifty micrograms of cell lysate was heated in 2× sample buffer for 5 min at 95°C, resolved in 7.5% or 12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore), and inhibited with 10 mmol/L Tris buffer (pH 7.5) (150 mmol/L NaCl, 0.05% Tween 20, and 5% dry milk) for 2 h. The membranes were incubated with anti–caspase-3 antibody (1 μg/mL). Isotype-matched irrelevant antibodies were used as controls. Membranes were then washed and blotted with horseradish peroxidase–protein A (Zymed Laboratories). After extensive washing with 10 mmol/L Tris-HCl buffer (pH 8.0) containing 150 mmol/L NaCl and 1% Tween 20, the membranes were developed in chemiluminescence reagent (NEN) and exposed to x-ray film.

Assessment of MPT in macrophages. Induction of MPT was assessed in macrophages, to evaluate the change in inner mitochondrial membrane potential, by measuring the amount of lipophilic cationic dye DiOC₆(3) retained within the mitochondria as a consequence of inner mitochondrial membrane potential [16]. MPT results in a loss of inner mitochondrial membrane potential, which decreases retention of DiOC₆(3) within the mitochondria. After performance of the experimental procedures, macrophages were pretreated with CHX (1 μg/mL), cultured in 6-well plates, and washed twice with PBS. The cells were then incubated for 20 min at 37°C with 1 mmol/L DiOC₆(3). Digitonin was added (to a concentration of 7.5 μmol/L) for 20 min, and the cells were washed 3 times with PBS, fixed with 1% formaldehyde for 20 min, and washed twice with PBS. Thereafter, the cells were dislodged from the plates by use of a rubber policeman and analyzed for fluorochrome incorporation by use of a FacSort flow cytometer (Becton Dickinson). The population of macrophages with reduced DiOC₆(3) staining was determined by setting the gate to allow separation of the cell population, with the population of macrophages with intact mitochondria showing high levels of staining with DiOC₆(3). At least 10⁵ events were analyzed. The percentage of macrophages with reduced DiOC₆(3) staining, within the total gated cell population, was determined.

Assessment of purinergic P2R function, by measurement of ethidium influx. Macrophages were cultured in 24-well plates, at a concentration of 5 × 10⁶ cells/mL, in RPMI and 10% human serum for 24 h; the cells were then infected with H37Ra (5 bacteria/cell) for 4 h, washed extensively, and incubated in the presence (7.5 μmol/L) or absence of CsA for another 24 h. Thereafter, 25 μmol/L ethidium and 1 mmol/L ATP (Sigma-Aldrich) were added. Ethidium uptake through a cation-selective channel was measured by FACS analysis every min until 5 min after the addition of ATP.

Statistical analysis. Results are expressed as mean ± SE. The data were analyzed by use of SigmaStat Statistical Software (version 1.0; Jandel Scientific), with the t test for normally distributed data with equal variances and the Mann-Whitney rank sum test for data populations with nonnormal distributions or unequal variances.

RESULTS

Induction of enhanced antimycobacterial activity in macrophages by inhibition of MPT. In macrophages infected with M. tuberculosis, opening of the PT pore, a large unselective pore in the inner mitochondrial membrane, causes loss of inner mitochondrial membrane potential and necrosis of macro-
This outcome is associated with rampant growth of the bacilli in the extracellular milieu and is enriched by the contents of the necrotic cells [7]. In addition to its function as an inhibitor of calcineurin, CsA has been shown to inhibit MPT [17]. We first investigated whether inhibition of MPT by CsA enhances defense against *M. tuberculosis*. To that end, macrophages were infected with either H37Rv or H37Ra (1 bacterium/cell) for 4 h and washed 4 times, and the macrophage cultures were then further incubated, in either the presence (5 μmol/L) or the absence of CsA. Cell cultures were harvested at different time points, and the viability of the bacteria was determined by assessing bacterial replication in cultures plated on 7H10 agar plates. Addition of CsA repressed the ability of both H37Ra and H37Rv to replicate (figure 1). Decreased uptake of *M. tuberculosis* in the presence of CsA was not the cause of this phenomenon, because the amount of *M. tuberculosis* phagocytosed in the presence of CsA and that phagocytosed in the absence of CsA were not significantly different. *M. tuberculosis* was incubated with macrophages for 1 h in the presence or absence of CsA. After 1 h, uptake of H37Rv, at an MOI of 1:1, was 0.38 × 10⁶ ± 0.15 × 10⁵ bacteria/mL in the absence of CsA and 0.41 ± 0.07 × 10⁵ bacteria/mL in the presence of CsA. Similar results were obtained with H37Ra (5 bacteria/cell) (data not shown). CsA had no direct growth-inhibitory effect on H37Ra when added to the culture medium in the absence of macrophages. At a starting concentration of 1.0 × 10⁷ ± 0.3 × 10⁶ bacteria/mL, the number of bacteria increased after 6 days, to 6.0 × 10⁷ ± 2.0 × 10⁷ bacteria/mL in the absence of CsA and to 5.8 × 10⁶ ± 1.9 × 10⁶ bacteria/mL in the presence of CsA. Because the effect of CsA on H37Ra was similar to the effect of CsA on H37Rv, we exclusively used H37Ra in further studies.

**Prevention of necrosis of macrophages by inhibition of MPT.**

In initial experiments, human alveolar macrophage cultures were infected with H37Ra (5 bacteria/cell) in the presence (5 μmol/L) or absence of CsA, and the cells were examined by light microscopy. *M. tuberculosis*-infected macrophage cultures had significant cell loss, which was inhibited by the presence of CsA (figure 2, lower 4 panels). We next measured cell death of human monocyte-derived macrophages infected with H37Ra, by PI staining (figure 2, upper right panel). After infection, the PI-positive macrophages increased from 2% at 24 h to 45% and 35% at 48 h and 72 h, respectively. The increase in numbers of PI-positive macrophages in the *M. tuberculosis*-infected macrophage cultures was also associated with increased release of DiOC₆(3) from the mitochondria (figure 2, upper left panel). CsA treatment was associated with reduced numbers of PI-positive cells and reduced release of DiOC₆(3). On the other hand, CsA had no effect on the number of TUNEL-positive macrophages in *M. tuberculosis*-infected macrophage cultures. The number of apoptotic cells after 48 h was 37% ± 4% in *M. tuberculosis*-infected macrophage cultures incubated in the absence of CsA and 34% ± 3% in *M. tuberculosis*-infected macrophage cultures incubated in the presence of CsA (n = 3; P = .15). Less than 3% of the cells in uninfected macrophage cultures were apoptotic at 0 and 48 h. These data demonstrate that H37Ra infection increases mitochondrial permeability and induces necrosis of infected macrophages.

Both events were inhibited by CsA, yet CsA did not inhibit manifestations of apoptotic cell death in these macrophage cultures. To exclude inhibition of calcineurin as the cause of the effect of CsA, we also tested the effect of the immunosuppressant FK508, a calcineurin inhibitor that lacks the capacity to inhibit MPT. In 3 experiments, FK506 was unable to rescue macrophages from necrosis, as determined by the 7-amino actinomycin necrosis assay (data not shown), indicating that inhibition of calcineurin by CsA is not involved in inhibition of necrosis.

**Inhibition of MPT and release of cytochrome c from the mitochondria in H37Ra-infected macrophages, by CsA.** The permeability of the mitochondria to DiOC₆(3) reflects a loss of inner mitochondrial membrane potential [17]. Loss of inner mitochondrial membrane potential correlates with opening of the MPT pore. In H37Ra-infected macrophages, CsA significantly suppressed the release of DiOC₆(3) from the mitochondria (figure 3A and 3B, a). Ruthenium red (RR) is a mitochondrial calcium uniporter inhibitor that decreases intramitochondrial concentrations of Ca²⁺ in treated cells [18]. We previously reported that RR dramatically increases MPT and necrosis in *M. tuberculosis*-infected macrophages [7]. In the present study, treatment of infected macrophages with RR did not affect the capacity of CsA to inhibit MPT (figure 3B, b). CsA also significantly down-regulated translocation of cytochrome c and necrosis in infected macrophages [11]. This outcome is associated with rampant growth of the bacilli in the extracellular milieu and is enriched by the contents of the necrotic cells [7].
In the present study, induction of caspase-3 by *M. tuberculosis* was not altered in the presence of CsA (figure 3B, d), presumably reflecting activation of the extrinsic apoptosis pathway by TNF-α [21].

Correlation between the capacity of CsA to control *M. tuberculosis* infection and functional purinergic P2Rs. Because CsA is able to restrict mycobacterial growth, we investigated whether CsA treatment affects the function of a known antimycobacterial mechanism in macrophages. Stimulation of the purinergic P2X receptors (P2XRs) has been reported to induce ATP-dependent killing of intracellular mycobacteria [22]. Signaling by purinergic P2Rs has also been shown to induce apoptosis of macrophages [23]. Although several groups have shown that apoptosis of *M. tuberculosis*-infected macrophages kills intracellular bacilli [2, 3, 12, 21], the antimycobacterial activity of ATP appears to be independent of induction of apoptosis [24]. Activation of P2XRs by ATP opens a cation-selective channel, which allows the entry of ethidium into cells [25, 26]. Because no clear differences could be detected between the antimycobacterial activity of CsA-treated macrophages in the presence or in the absence of ATP, we measured ethidium uptake, a functional correlate of P2XRs. Figure 4 shows that the capacity to take up ethidium after stimulation with ATP was significantly lower in macrophages infected with H37Ra (24 h) than in uninfected macrophages and macrophages infected with H37Ra and treated with CsA. Our findings demonstrate that impaired uptake of *M. tuberculosis*-infected macrophages by ATP-dependent ethidium is restored by CsA. It remains to be determined whether the capacity of CsA to restore P2XR function in this setting is associated with the maintenance of mitochondrial integrity or whether it is the consequence of a different effect of CsA.

**DISCUSSION**

The involvement of the mitochondria in programmed cell death has now been firmly established on the basis of the finding that redistribution of apoptogenic factors (such as cytochrome c and Smac/Diablo) from the mitochondrial intermembrane space into the cytosol causes activation of caspase, followed by cell death [27–29]. It is thought that the relative rate of the bioenergetic mitochondrial collapse during cell death determines whether cell death resembles necrosis or apoptosis [11]. In the present study, CsA was used to inhibit MPT by *M. tuberculosis*-infected macrophages, to investigate whether prevention of mitochondrial membrane damage increases the intrinsic antimycobacterial activity of these cells. Inhibition of opening of the PT pore is a highly selective function of CsA, because it apparently affects no other mitochondrial functions [30]. We have shown here that, in H37Ra-infected macrophages, CsA prevents redistribution of cytochrome c from the mitochondria to the cytosol, inhibits mitochondrial collapse during cell death, resulting in sustained increases of cytosolic calcium [20].

Cytoplasmic translocation of cytochrome c results in activation of caspase-9, with subsequent activation of downstream caspases [19]. Release of cytochrome c also amplifies apoptotic signaling through binding to inositol triphosphate receptors in the endoplasmatic reticulum [1, 4, 5], resulting in sustained increases of cytosolic calcium [20].

**Figure 2.** Upper left panel, Inhibition of death of macrophages infected with H37Ra, determined by propidium iodide (PI) staining of uninfected macrophages (1) in the absence of cyclosporin A (CsA) (no additives), (2) in the presence (5 μmol/L) of CsA, (3) infected with H37Ra (5 bacteria/cell) (H37Ra), or (4) infected with H37Ra and treated with CsA (H37Ra + CsA), after 24, 48, and 72 h. The nos. of PI-positive cells at 48 and 72 h, in cultures of *Mycobacterium tuberculosis*-infected macrophages incubated in the presence or absence of CsA, were significantly different (n = 3, P = .005, t test). Upper right panel, Inhibition of mitochondrial permeability transition (MPT) by CsA, measured in the same experiment, correlated with inhibition of cell death. Macrophages were analyzed, by flow cytometry, for retention of DiOC(3) in the mitochondria as an indicator of MPT, by gating the cells containing mitochondria with reduced activity of these cells. Inhibition of opening of the PT pore is a highly selective function of CsA, because it apparently affects no other mitochondrial functions [30]. We have shown here that, in H37Ra-infected macrophages, CsA prevents redistribution of cytochrome c from the mitochondria to the cytosol, inhibits mitochondrial collapse during cell death, resulting in sustained increases of cytosolic calcium [20].
Figure 3.  

A. Inhibition of release of cationic dye from the mitochondria of H37Ra-infected macrophages, by cyclosporin A (CsA). In a typical experiment, cells preloaded with DiOC6(3) and infected for 48 h with H37Ra (5 bacteria/cell) were cultured in the presence (1 or 5 μmol/L) or absence of CsA. Retention of DiOC6(3) in the mitochondria of macrophages was measured by use of a fluorescence-activated cell sorter. 

B. a, Significantly lower retention of cationic dye in the mitochondria of Mycobacterium tuberculosis–infected macrophages, compared with that in the mitochondria of M. tuberculosis–infected macrophages incubated in the presence of CsA (p = 3; P = .004 for 1 μmol/L CsA; P = .005 for 5 μmol/L CsA). The ordinate shows the percentage of cells with reduced retention of dye in the mitochondria. b, Inhibition of mitochondrial permeability transition in the mitochondria of RR-treated macrophage cultures infected with M. tuberculosis, by CsA. DiOC6(3)-preloaded macrophages infected with M. tuberculosis in the presence of 5 μg/mL RR for 48 h were incubated in the presence (5 μmol/L) or absence of CsA. Retention of cationic dye in RR-incubated macrophages infected with M. tuberculosis was significantly inhibited by CsA (p = 3; P = .005). 

c, Inhibition of release of cytochrome c from the mitochondria of M. tuberculosis–infected macrophages, by CsA. Macrophages were infected for 48 h with H37Ra (5 bacteria/cell) in the presence (5 μmol/L) or absence of CsA. Levels of cytochrome c in the mitochondria and in the cytosol were determined by Western blotting. Antiactin antibody was used as a loading control. 

d, No effect of CsA on caspase-3 activation in M. tuberculosis–infected macrophages. Macrophages were infected (5 bacteria/cell) or not infected with M. tuberculosis (5 μmol/L) or absence of CsA. After 24 h, the cells were examined, by Western blotting, for procaspase-3 (32 kDa) degradation. Antiactin antibody was used in parallel as a loading control.
to mitochondrion damage, and inhibits down-regulation of P2XR function, a consequence of mycobacterial infection.

CsA is thought to bind to the mitochondrial matrix component CyP-D [8, 9]. CyP-D associated with CsA is thought to be unable to bind to the intramitochondrial adenine nucleotide translocator, a reaction that results in irreversible opening of the MTP [31]. This high-conductance mitochondrial inner membrane channel then participates in the formation of the PT pore [8], which remains closed in the presence of CsA. CsA is also a potent inhibitor of calcineurin and causes immunosuppression by preventing calcineurin-mediated activation of the transcription factor nuclear factor of activated T cells [32]. However, experiments using FK506 have indicated that inhibition of calcineurin by CsA is not implicated in the inhibition of MPT. The inhibitory effects of CsA on activation of T lymphocytes clearly limit its potential usefulness as an enhancer of innate antimycobacterial activity of macrophages. The CsA derivative N-methyl-val-4–CsA (NMV-CsA) is unable to inhibit calcineurin but retains CyP-D binding activity and the capacity to inhibit MPT [33]. Although NMV-CsA is not presently available, our data suggest that derivatives of CsA might be useful drugs to augment innate host immune defense in tuberculosis disease, to prevent infection with *M. tuberculosis*, or to inhibit spread of the infection.

The effect of CsA resembles that of certain members of the Bcl-2 family that affect mitochondrial permeability to intermembrane space proteins through interaction with mitochondrial membrane components [34], thereby preventing mitochondrial damage [35]. There is evidence that pro- and antiapoptotic members of the Bcl-2 family function by binding to VDAC, the voltage-dependent anion channel situated in the outer mitochondrial membrane, which is also associated with MPT [36]. They either accelerate opening of VDAC, in the case of the proapoptotic proteins Bax and Bak, or close the pore, in the case of the antiapoptotic protein Bcl-x [37] or the macrophage protein Mcl-1. Mcl-1 was recently found to be up-regulated in THP1 cells during infection with *M. tuberculosis*, causing suppression of apoptosis [38]. In the absence of CsA, a considerable proportion of macrophages infected with H37Ra undergo cell death that resembles necrosis and is characterized by PI-positive cells. Necrotic cells release intracellular components into the surrounding medium because of loss of cell membrane integrity, which promotes inflammatory responses [39] and permits extracellular spread of infection. Part of the inflammatory response is an increased tissue influx of neutrophil granulocytes and macrophages due to the action of chemokines [40]. This mechanism may be part of an antibacterial defense response [41], but, in active pulmonary tuberculosis, exacerbated necrosis might also be harmful by contributing to morbidity and to disease transmission. A consequence of the action of CsA is inhibition of the production of chemokines, such as growth-related protein α, by the necrosis of macrophages, because accumulation of necrotic cell material responsible for the induction of chemokine production is significantly restricted.

We also detected that infection with H37Ra abrogates the function of purinergic P2Rs on the macrophage. The defective P2XR function can be reconstituted with CsA. Whether stabilization of inner mitochondrial membrane potential is required for proper P2XR function is not known. P2XR is thought to be of critical importance in the defense of macrophages against mycobacterial infection [12], and macrophages from P2XR−/− mice are unable to kill mycobacteria [13]. Because the presence of CsA alone in H37Ra-infected macrophages leads to high antimycobacterial activity, it is possible that high concentrations of extracellular ATP essential for induction of antimycobacterial activity by P2XR exist in apoptotic macrophages. Accordingly, P2XR might be activated in H37Ra-infected macrophage cultures by extracellular ATP released from damaged cells undergoing necrosis [42], from exosomes.
cytotic vesicles or granules present in secretory cells [43], or from macrophages after stimulation with bacterial products [44].

In summary, we found that, by inhibiting MPT, CsA inhibits necrosis of M. tuberculosis–infected macrophages. In contrast, expression of apoptosis was not affected. This situation might enhance the antimycobacterial mechanisms linked to apoptosis by delaying the onset of secondary necrosis in dying cells. We also found that CsA maintains the functional activity of P2XR in macrophages infected with H37Ra, and signaling by P2XR has been linked to killing of mycobacteria in macrophages. Our data suggest that CsA not only slows the rate of bioenergetic mitochondrial collapse but seems to maintain P2XR function in infected macrophages. Future studies are needed to determine whether events leading to cell death are required for optimal expression of antimycobacterial activity of macrophages and to elucidate the opposing effects of CsA and M. tuberculosis on P2XR function.

References
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