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# The 19-kDa *Mycobacterium tuberculosis* Protein Induces Macrophage Apoptosis Through Toll-Like Receptor-2<sup>1</sup>

Martín López,<sup>2\*</sup> Laura M. Sly,<sup>2\*</sup> Yvonne Luu,<sup>\*</sup> Douglas Young,<sup>‡</sup> Howard Cooper,<sup>‡</sup> and Neil E. Reiner<sup>3\*†</sup>

Macrophages infected with *Mycobacterium tuberculosis* undergo increased rates of apoptosis. Important objectives are to define the microbial factors that cause apoptosis, the mechanisms involved and the impact on infection. The 19-kDa *M. tuberculosis* glycolipoprotein (p19) is both cell wall-associated and secreted and is a candidate virulence factor. We investigated the potential of recombinant, His-tagged p19 lacking the secretion/acylation signal to induce macrophage apoptosis. The TUNEL assay and annexin V binding to membrane phosphatidylserine were used to measure apoptosis. The results show that p19 does act to induce apoptosis in differentiated THP-1 cells and monocyte-derived macrophages and that this effect is both dose- and time-dependent. Furthermore, this effect of p19 is Toll-like receptor (TLR)-2-mediated because preincubation of either THP-1 cells or TLR-2-expressing CHO cells with anti-TLR-2 mAb inhibited apoptosis induced by p19. Apoptosis of macrophages in response to p19 was found to be caspase-8 dependent and caspase-9 independent consistent with a transmembrane pathway signaling cell death through TLR-2. The viability of *M. tuberculosis* in cells undergoing apoptosis induced by p19 was significantly reduced suggesting the possibility that this may favor containment of infection. Although native p19 is a mycobacterial glycolipoprotein, based upon the use of recombinant p19 where the acylation signal had been removed, we conclude that it is the polypeptide component of p19 that is responsible for signaling through TLR-2 and that the lipid moiety is not required. *The Journal of Immunology*, 2003, 170: 2409–2416.

Almost one-third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of pulmonary tuberculosis, and estimates from the World Health Organization suggest that there are ~8 million new cases and 3 million deaths worldwide from this disease each year (1, 2). Tuberculosis remains a serious global health problem in part because of our lack of a detailed understanding of its pathogenesis.

During the early stages of infection with *M. tuberculosis*, the extent of bacterial survival and proliferation is mainly determined by the efficacy of the innate immune response with macrophages as the main effector cells (3, 4). Concomitant with phagocytosis of *M. tuberculosis*, macrophages may express several antimicrobial mechanisms to control intracellular replication of bacilli, including the production of reactive oxygen and nitrogen intermediates as well as nonoxidative mechanisms (5, 6). Despite the multiplicity of antimicrobial mechanisms available to its host cell, *M. tuberculosis* shows a remarkable ability to survive and this may involve interference with critical macrophage functions that are normally required to successfully respond to infection (7–12). The mecha-

nisms used by *M. tuberculosis* to modify macrophage functional properties have not been fully characterized.

A variety of mycobacterial species (13–17) have been shown to be capable of causing macrophages to undergo increased rates of apoptosis in vitro. Consequently, considerable interest has emerged recently in the potential role of macrophage apoptosis in host defense against mycobacterial infection. In vitro infection of both monocytes and alveolar macrophages with *M. tuberculosis* has been shown to result in apoptosis, and an increased frequency of apoptosis has been observed in alveolar macrophages recovered from patients with pulmonary tuberculosis (18–20). Whether or not exposure of macrophages to *M. tuberculosis* in vitro results in apoptosis may be influenced by either the duration or multiplicity of infection or both. For example, it has been reported that apoptosis occurs early after infection with *M. tuberculosis* and may be directly proportional to the multiplicity of infection (14). The latter is consistent with the finding that infection with low numbers of viable bacilli prevented spontaneously occurring apoptosis in monocytes (21). It has also been reported that in comparison to relatively avirulent strains, infection of macrophages with virulent *M. tuberculosis* results in less apoptosis (20, 22).

Although the evidence available appears to favor the conclusion that *M. tuberculosis* can induce macrophage apoptosis, important unresolved questions remain including the identities of the responsible microbial factors, the underlying apoptotic mechanism involved, and the impact of apoptosis on the course of infection. With regard to mechanisms, advances in understanding how *M. tuberculosis* interacts with macrophages suggest that apoptosis may involve signaling through mammalian Toll-like receptors (TLRs)<sup>4</sup> and TLR-2 in particular. Thus, recent findings indicate that *M. tuberculosis* and various subcellular fractions derived from

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<sup>4</sup> Abbreviations used in this paper: TLR, Toll-like receptor; p19, 19-kDa *M. tuberculosis* glycolipoprotein; PS, phosphatidylserine; CHO, Chinese hamster ovary; PCD, programmed cell death; MDM, monocyte-derived macrophages.

it are recognized by mammalian TLRs. Members of this family of innate, pattern-recognition receptors have been shown to function as macrophage signaling receptors that respond to a variety of molecularly diverse bacterial products including lipoproteins (23–26), LPS (27), flagellins (28), and CpG dinucleotide repeats (29). Two members of this large family, TLR-2 and TLR-4, have been shown to activate intracellular signaling in response to *M. tuberculosis* or its products (30–32). For example, it has been reported that both viable *M. tuberculosis* as well as subcellular fractions contain ligands able to activate cells through either TLR-2 or TLR-4 (30). These factors appeared to operate independent of CD14 and were distinct from mycobacterial lipoarabinomannan. MyD88, an intracellular adapter protein known to be involved in membrane proximal TLR signaling, has also been shown to be required for signaling through TLR-2 in response to either intact *M. tuberculosis* or its lipid-enriched fractions leading to the production of TNF- $\alpha$  (31).

Identification of specific factors from *M. tuberculosis* responsible for inducing apoptosis will provide insight into pathogenesis and may suggest novel strategies for disease prevention or treatment. The recent finding that synthetic bacterial lipoproteins can induce apoptosis through TLR-2 (33) suggested the possibility that the 19-kDa mycobacterial lipoprotein (p19) may be an *M. tuberculosis* apoptosis-inducing factor. p19 is an abundantly expressed cell wall-associated and secreted glycolipoprotein. It has a highly conserved six-residue consensus sequence for lipidation adjacent to the hydrophobic signal peptide. The precursor undergoes post-translational acylation and subsequent cleavage of the signal peptide before secretion across the cell membrane (34, 35). Incubation of human macrophages with purified, native p19 has been shown to induce IL-12 production through TLR-2 (23), and exposure of murine Raw264.7 cells infected with *M. tuberculosis* to p19 resulted in reduced viability of intracellular bacilli also dependent on TLR-2 (36).

In the present study we examined whether p19 may act as an extracellular proapoptotic factor and if so, whether this is TLR-2 dependent. Based upon results of two independent measurements for programmed cell death, namely the TUNEL assay and annexin V binding to phosphatidylserine (PS) exposed on the outer leaflet of the cell membrane, we present evidence that p19 does act to induce apoptosis in cells of the human monocytic cell line THP-1 and human monocyte-derived macrophages (MDMs). Furthermore, this effect of p19 appears to be TLR-2-mediated because preincubation of either THP-1 cells or TLR-2-expressing Chinese hamster ovary (CHO) cells with a blocking anti-TLR-2 mAb inhibited apoptosis induced by p19. Consistent with a signaling pathway activated by TLR-2, p19-induced apoptosis was also found to involve the sequential activation of caspase-8 and caspase-3. Finally, although p19 is a mycobacterial glycolipoprotein, our results using recombinant protein expressed in *Escherichia coli* indicate that it is the polypeptide component of p19 that is responsible for signaling through TLR-2 for apoptosis.

## Materials and Methods

### Specialized reagents and Abs

RPMI 1640 and polymyxin B resin (P-1411) were from Sigma-Aldrich Canada (Oakville, Ontario, Canada). DMEM, nonessential amino acids, and streptomycin/penicillin were from StemCell Technologies, Terry Fox Laboratory (Vancouver, British Columbia, Canada). FBS was from HyClone Laboratories (Logan, UT). Middlebrook 7H9, 7H10, and OADC were from Difco (Detroit, MI). The Kinyoun staining kit was from BBL Microbiology Systems (Cockeysville, MD). Caspase inhibitors Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), and Z-FA-FMK (negative control) were from Enzyme Systems Products (Livermore, CA). Caspase inhibitor Z-DQMD-FMK (caspase-3 inhibitor V) and

actinomycin D were from Calbiochem (San Diego, CA). Stock solutions of caspase inhibitors were prepared in DMSO and stored at  $-20^{\circ}\text{C}$ . Fluorescein FragEL DNA fragmentation detection kit was from Oncogene Research Products (Boston, MA). Annexin V-PE was from BD Pharmingen (Mississauga, Ontario, Canada).

### Culture and manipulation of THP-1 and CHO cell lines

Cells of the human promonocytic cell line THP-1 were grown at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 20  $\mu\text{M}$  2-ME, 10% FBS, and streptomycin and penicillin as described previously (37). Monocytic differentiation was induced with PMA at 20 ng/ml final concentration (overnight incubation). CHO cells were grown at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, streptomycin and penicillin. Wild-type CHO cells and CHO cell transfectants stably expressing either Fc $\gamma$ R or TLR-2 were generously provided by Dr. D. Golenbock (Boston University, Boston, MA).

### Purification and culture of MDMs

Purification of monocytes was performed by Ficoll gradient and macrophages were further selected for adherence as described previously (6).

### Purification of recombinant His-tagged p19

The mature coding region of p19 from *M. tuberculosis* had been previously cloned into the *Sma*I site of the Qiagen (Mississauga, Ontario, Canada) pQE-30 plasmid vector (38). p19 protein was produced as a His-tagged recombinant protein containing six His residues at its amino terminal end. This recombinant form of p19 lacks the first four amino acids that constitute the site of lipid modification. This modification promotes its solubility during the extraction procedure. His-tagged p19 was purified from *E. coli* using the cobalt-based TALON Metal Affinity Resin (Clontech Laboratories, Palo Alto, CA). Purification of the protein was monitored by SDS-PAGE (10%) with Coomassie brilliant blue R-250 and silver staining and by immunoblotting using a mAb specific for p19 (provided by Dr. J. Belisle, Colorado State University, Fort Collins, CO).

### Incubation of macrophages with *M. tuberculosis* p19

PMA-differentiated THP-1 cells or MDMs were seeded in 24-well flat-bottom tissue culture plates at a density of  $1-2 \times 10^5$  cells per well and allowed to adhere and differentiate at  $37^{\circ}\text{C}$ . Cells were washed and medium was replaced 3 h before any treatment. Cells were incubated with His-tagged p19 in a range of concentrations from 5 to 200 ng/ml for 2 h. After washing, cells were incubated at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for up to 24 h. Culture supernatants and cells were collected and washed with cold PBS. Cell apoptosis was evaluated using both the TUNEL assay and binding of annexin V to follow PS exposure on the outer leaflet of the cell membrane. To control for the possibility of nonspecific effects of the His tag itself, control cells were incubated with His-tagged  $\beta$ -galactosidase. Moreover, because p19 was produced in *E. coli*, we controlled for the possibility of nonspecific effects of LPS by including treatment groups incubated with either heat-inactivated p19 alone or with p19 that had been pretreated with polymyxin B. Further, we used native p19 to directly compare its effects in these assays.

### Treatment of CHO-TLR-2 cells with p19

CHO cells expressing TLR-2 were seeded in 24-well plates to a density of  $\sim 1 \times 10^5$  cells per well at the time of treatment. Cells were incubated with His-tagged p19 (100 ng/ml) for 4 h. After washing, cells were incubated at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for an additional 20 h. Cells were collected and washed with cold PBS. Annexin V binding to CHO-TLR-2 cells was monitored to measure cell apoptosis. To control for receptor specificity, CHO cells stably expressing Fc $\gamma$ R and CHO cells containing only the plasmid vector were treated in parallel. To control for reagent specificity, parallel cultures of CHO cells were incubated under identical conditions except with His-tagged  $\beta$ -galactosidase replacing His-tagged p19.

### Incubation of THP-1 and CHO cells with p19 in the presence of mAb against TLR-2

PMA-differentiated THP-1 cells or CHO cells were seeded in 24-well flat-bottom plates as described previously. Cells were incubated with either human TLR-2-specific mAb 2392 (25  $\mu\text{g}/\text{ml}$ ) or an isotype-matched irrelevant mAb for 1 h before the addition of His-tagged p19 at a final concentration 100 ng/ml. Incubation of THP-1 and CHO cells with p19 was for 2 or 4 h, respectively. Cells were then washed, the medium was replaced, and cells were incubated for a total of 24 h at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . Apoptosis was measured by annexin V binding.

### Incubation of THP-1 cells with caspase inhibitors

PMA-differentiated THP-1 cells were incubated with His-tagged p19 in the presence or absence of cell-permeable, irreversible inhibitors of caspase-8, caspase-9, or caspase-3. Inhibitors were added to the cells 2 h before the addition of p19 (50 ng/ml). Incubation with p19 was for 2 h at which point the cells were washed, the medium was replaced, and incubation was continued for an additional 20 h before the apoptosis assay.

### *M. tuberculosis* infections and p19 treatment

THP-1 cells or MDMs were incubated with 50 ng/ml p19 either before or after infection with *M. tuberculosis* strain H37Rv. Cells were maintained at a density of  $5\text{--}10 \times 10^5$  cells per ml. *M. tuberculosis* was grown to late log phase in Middlebrook 7H9 with OADC. Aliquots were frozen at  $-70^\circ\text{C}$  until used. Representative samples were thawed and CFUs per ml were determined by plating on Middlebrook 7H10 with OADC. Cells were infected as described previously (6). Briefly, THP-1 cells were seeded in 6-well, flat-bottom cell culture plates and allowed to adhere and differentiate at  $37^\circ\text{C}$  overnight in the presence of PMA. MDMs were plated and allowed to differentiate for 3 days before infection. Cells were washed and medium was replaced 4 h before the addition of bacteria. Bacteria were opsonized by incubating with 50% fresh human serum in RPMI for 30 min before addition to cells. Bacteria were added at infection ratio of 50:1 for THP-1 cells and 5:1 for MDMs. Infection was evaluated by measuring CFUs. After coinoculation of bacteria with cells, the monolayers were washed three times with HBSS to remove non-ingested bacilli and the medium was replenished. THP-1 cells were collected at the time points indicated and evaluated for apoptosis by TUNEL assay and by annexin V binding assays. Intracellular survival of bacilli was assessed in THP-1 cells and MDMs by recovery of CFUs.

### Determination of CFUs

Enumeration of CFUs was performed as described previously (6). Briefly, bacilli were plated immediately after infection of cells ( $t = 0$ ) and at either day 4 or day 7 after infection. Organisms were released in cold PBS, 0.1% Triton X-100, serially diluted in Middlebrook 7H9 with OADC, and 20  $\mu\text{l}$  of three dilutions were plated in triplicate on Middlebrook 7H10 agar. CFUs were counted after 14 days of incubation at  $37^\circ\text{C}$ , and plates were maintained for 21 days to ensure that no additional CFUs appeared.

### Apoptosis assays

Apoptosis was measured by either TUNEL assay or annexin V staining. TUNEL assays were performed using the Fluorescein FragEL DNA fragmentation kit according to the manufacturer's instructions. Cells were considered apoptotic if they were TUNEL positive (green fluorescent nuclear staining). Positive controls included incubations of cells with actinomycin D. For annexin V-PE binding, cells were collected and washed with cold PBS and resuspended in annexin V-PE binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ). An aliquot of 100  $\mu\text{l}$  was removed and mixed with 5  $\mu\text{l}$  of annexin V-PE. The mixture was vortexed and incubated for 15 min at room temperature in the dark. The cells were then washed once with binding buffer and the volume was increased to 500  $\mu\text{l}$  with binding buffer for analysis by flow cytometry.

### Statistical analyses

Data presented are expressed as mean  $\pm$  SD. Statistical analyses for annexin V-PE binding and TUNEL assays were performed using a Student *t* test, and values for recovery of CFUs were compared by one-way ANOVA at each time point. Differences were considered significant at  $p < 0.05$ .

## Results

### p19-induced apoptosis

PMA-differentiated THP-1 cells or MDMs were incubated with a range of concentrations of recombinant p19. Apoptosis was assessed by detection of PS exposure on the outer leaflet of the cell membrane by annexin V binding and flow cytometric analysis or by TUNEL assay for THP-1 cells. As shown in Fig. 1 (*left side*), increased levels of apoptosis were apparent with as little as 20 ng/ml p19 and continued to increase through 200 ng/ml, the maximum concentration examined. The basal frequency of apoptosis in untreated cells was in the range of 5% for THP-1 cells and 3% for MDMs. At 50 ng/ml p19, apoptosis increased  $\sim$ 3-fold to 27% in THP-1 cells and to 43% in MDMs. A kinetic analysis conducted at

a concentration 50 ng/ml p19 showed that significant apoptosis was detectable as early as 30 min after exposure and continued to increase through 24 h (Fig. 1, graphs, *right side*).

### p19-induced apoptosis is not due to the His tag nor LPS contamination

Recombinant p19 used in this study was expressed in *E. coli* as a His-tagged fusion protein. To control for nonspecific effects, His-tagged  $\beta$ -galactosidase was used in parallel. As shown in Fig. 2A, the frequency of annexin V-PE-positive cells after incubation with the  $\beta$ -galactosidase fusion protein was similar to that observed for untreated control cells. This result indicated that the His tag was not likely contributing to the effects observed for p19.

To examine whether LPS contamination might be contributing to apoptosis induction by p19, two approaches were used. These methods involved exposure of the recombinant protein to either heat treatment or to polymyxin B before addition to cells. As shown in Fig. 2, B and C, exogenous LPS when added to THP-1 cells was moderately proapoptotic and was resistant to heat treatment and sensitive to polymyxin B. In contrast, the proapoptotic effects of both the His-tagged p19 and native p19 protein were completely abrogated after heat treatment and were polymyxin B-resistant. These findings indicate that LPS contamination was not contributing to the apoptosis observed in cells exposed to recombinant p19, and that effect is similar to that seen with native p19.

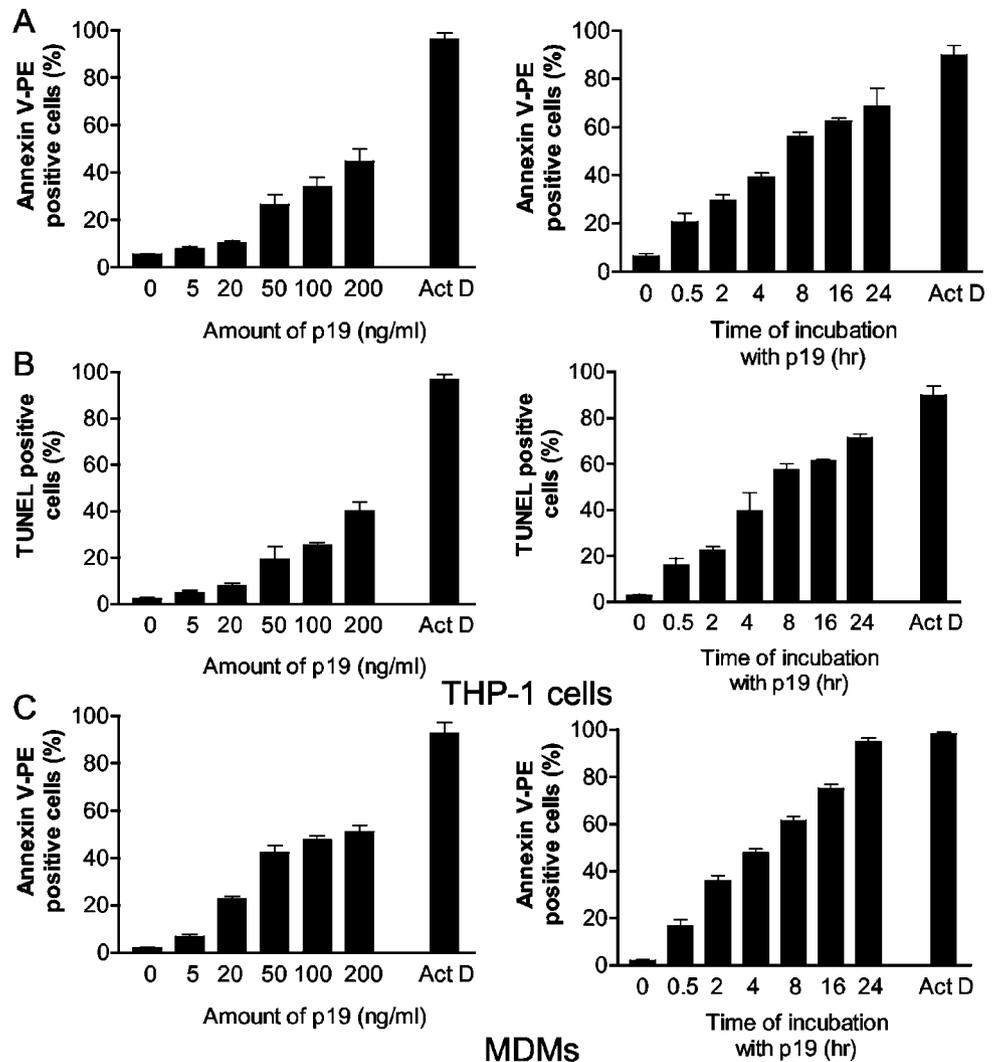
### p19 signaling for apoptosis induction is TLR-2 dependent

We examined the role of TLR-2 in p19-induced apoptosis using CHO cells stably transfected with human TLR-2. As shown in Fig. 3A, when compared with control untreated cells, incubation of CHO-TLR-2 cells with His-tagged p19 resulted in an  $\sim$ 2-fold increase in annexin V-PE binding consistent with apoptosis induction. In contrast, p19 was unable to induce apoptosis in either wild-type CHO cells or in CHO cells expressing Fc $\gamma$ R (both lacking TLR-2). Moreover, no effect was observed when TLR-2 CHO cells were incubated with His-tagged  $\beta$ -galactosidase.

To examine further the requirement for TLR-2, both THP-1 cells and CHO cells expressing TLR-2 cells were incubated with p19 in the presence or absence of mAb 2392 specific for human TLR-2. As shown in Fig. 3, B and C, apoptosis induced in THP-1 cells by p19 was completely abrogated by mAb 2392. Although not as complete, mAb treatment also significantly reduced the extent of p19-induced apoptosis in CHO cells expressing TLR-2. For both THP-1 cells and CHO cells expressing TLR-2, an isotype matched, irrelevant mAb (i/c) did not affect apoptosis induction by p19. Taken together, these findings indicate that p19-induced apoptosis is initiated by transmembrane signaling through TLR-2.

### p19-induced apoptosis is caspase-8 dependent

Evidence from other systems has suggested that TLR-2-induced apoptosis proceeds through a pathway involving caspase-8 (33). To examine whether p19-induced apoptosis was caspase-8 dependent, before exposure to p19, PMA-differentiated THP-1 cells were incubated with Z-IETD-FMK, a cell membrane permeable caspase-8 inhibitor. Significant inhibition of p19-induced apoptosis was observed in cells preincubated with 75  $\mu\text{M}$  of Z-IETD-FMK (Fig. 4). Z-FA-FMK peptide, which lacks the residues necessary for caspase inhibition, was used as a control (c) and did not have any effect on apoptosis induction. Like the caspase-8 inhibitor, a caspase-3 inhibitor peptide (Z-DQMD-FMP) also abrogated p19-induced apoptosis (Fig. 4). In contrast, preincubation of cells with a caspase-9 inhibitor did not attenuate p19-induced apoptosis. p19-Induced apoptosis was further distinguished from actinomycin D in that apoptosis induced by the latter was resistant to the



**FIGURE 1.** *M. tuberculosis* p19 induces apoptosis in macrophages. PMA-differentiated THP-1 cells or MDMs were incubated with either a range of concentrations of p19 for 2 h (graphs, left side) or for varying lengths of time with 50 ng/ml recombinant protein (graphs, right side). Apoptosis was analyzed by measuring membrane exposure of PS using annexin V by flow cytometry for THP-1 cells (A) or MDMs (B) or by TUNEL assay of THP-1 cells (C). Incubation of cells with actinomycin D was used as a positive control for apoptosis. The results shown are the means  $\pm$  SD of three independent experiments each performed in duplicate.

caspase-8 inhibitor but was sensitive to either the caspase-9 inhibitor or caspase-3 inhibitor peptide.

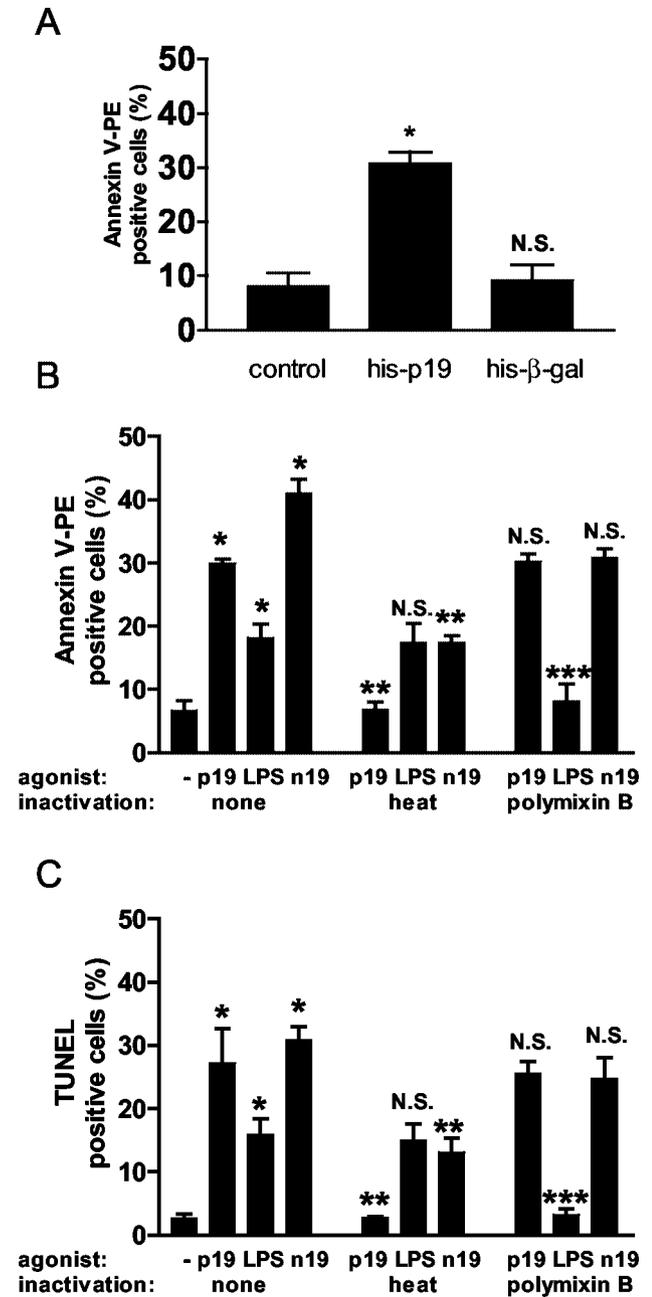
#### Effect of p19 treatment of THP-1 cells on intracellular growth of *M. tuberculosis*

To investigate potential effects of p19-induced apoptosis on survival of *M. tuberculosis*, THP-1 cells were incubated with p19 either before or following infection. Measurement of annexin V-PE binding, TUNEL assays, and determination of CFUs were performed. CFUs recovered from similarly treated MDMs were also evaluated. As shown in Fig. 5, A and B, infection by itself resulted in a modest but not significant increase in annexin V binding or TUNEL positivity compared with control (uninfected cell, no p19 treatment) at 24 h after infection. The effect of p19 treatment of noninfected cells whether “pre” or “post” was different from the corresponding noninfected control with  $p < 0.01$ . Moreover, either pre- or posttreatment of infected cells with p19 resulted in increased annexin V binding that was different from the corresponding p19-treated, noninfected control group (pre 0.8% level and post 2.5% level), suggesting the occurrence of an interaction between infection and p19 treatment. The impact of p19-induced apoptosis on the survival of *M. tuberculosis* was examined. The results (Fig. 5, C and D) show that incubation of THP-1 cells or MDMs, respectively, with p19 either before or after infection resulted in a significant reduction in recovery of CFUs and

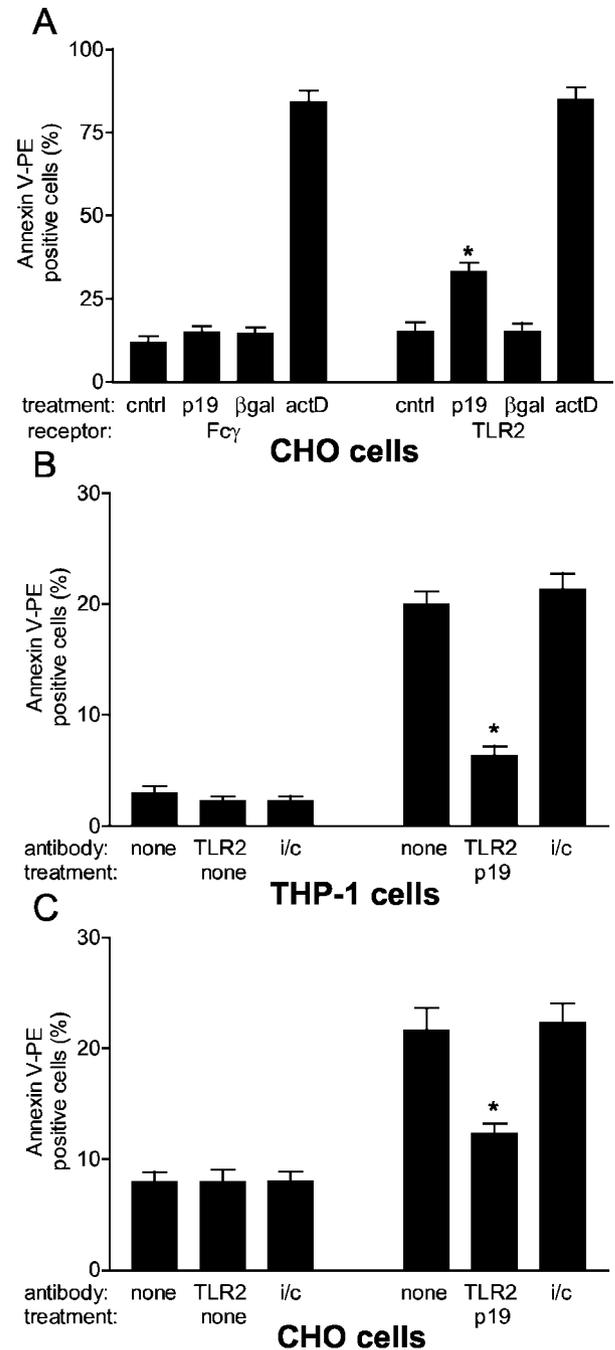
this effect was more dramatic when recovery of viable bacilli was assessed at day 7 following p19 treatment ( $p < 0.03$  at day 4 and  $p < 0.01$  at day 7).

#### Discussion

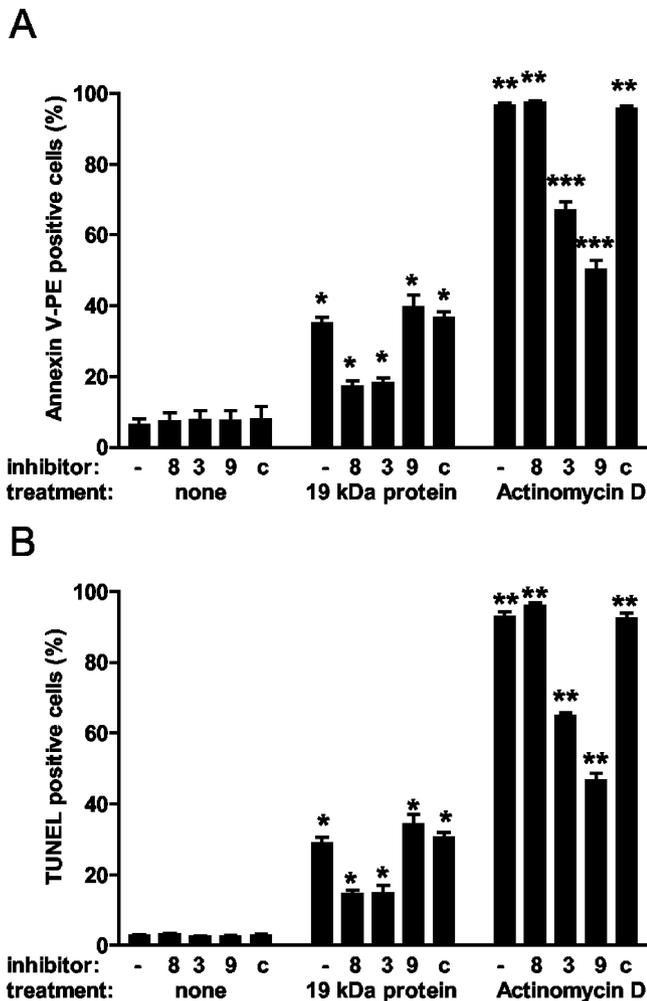
Apoptosis or programmed cell death (PCD) is a tightly regulated suicide program leading to cell death, and as such it plays a central role in maintaining the integrity and homeostasis of multicellular organisms (reviewed in Ref. 39). PCD involves the proteolytic action of caspases, a family of cysteine proteases that cleave a large group of cellular protein substrates on the C-terminal side of aspartic acid. Two major pathways of caspase activation leading to PCD have been defined. One pathway is initiated at the cell surface through transmembrane death receptors such as Fas/CD95 coupled to activation of caspase-8. In some cell types such as lymphoid cells, this is sufficient to bring about the direct and sequential activation of “executioner” caspase-3 and the cleavage of cellular substrates. In other cell types, death receptor signaling may result in caspase-8 activation that is insufficient to activate caspase-3 directly, and in these instances the mitochondrial pathway of PCD may be recruited. Here, activated caspase-8 cleaves proapoptotic Bcl-2 family member Bid to generate a fragment that triggers the release of cytochrome *c* from mitochondria resulting in the sequential activation of caspase-9 and caspase-3. A variety of factors acting intracellularly, such as spindle poisons, which are agents



**FIGURE 2.** p19-Induced apoptosis is not due to His tag or LPS contamination. **A**, THP-1 cells were incubated with His-tagged β-galactosidase as control for specificity. Cells were incubated with equal amounts of His-tagged β-galactosidase or His-tagged p19. \*,  $p < 0.01$  for His p19 treatment compared with untreated or His β-galactosidase-treated cells; N.S., not significantly different from control cell. **B** and **C**, THP-1 cells were incubated with recombinant p19, LPS or native p19. Before addition to cells (50 ng/ml for 2 h), His-tagged p19 was either subjected to boiling for 1 h or treated with polymyxin B resin. For the latter, 100 μl of polymyxin bead slurry was added to 100 μl of recombinant p19 preparation and incubated at 4°C for 1 h. Beads were removed and the supernatant was assayed for protein content and used for apoptosis induction. \*,  $p < 0.03$  comparing native or recombinant p19 treatment or LPS treatment to untreated cells; \*\*,  $p < 0.01$  comparing heat inactivated native or recombinant p19 treatment to p19 treatment; \*\*\*,  $p < 0.05$  comparing polymyxin B-treated LPS to LPS; N.S., heat treated LPS and polymyxin B-treated native or recombinant p19 were not significantly different from LPS or p19 treatments, respectively. The results shown are the means ± SD of three independent experiments performed in triplicate.



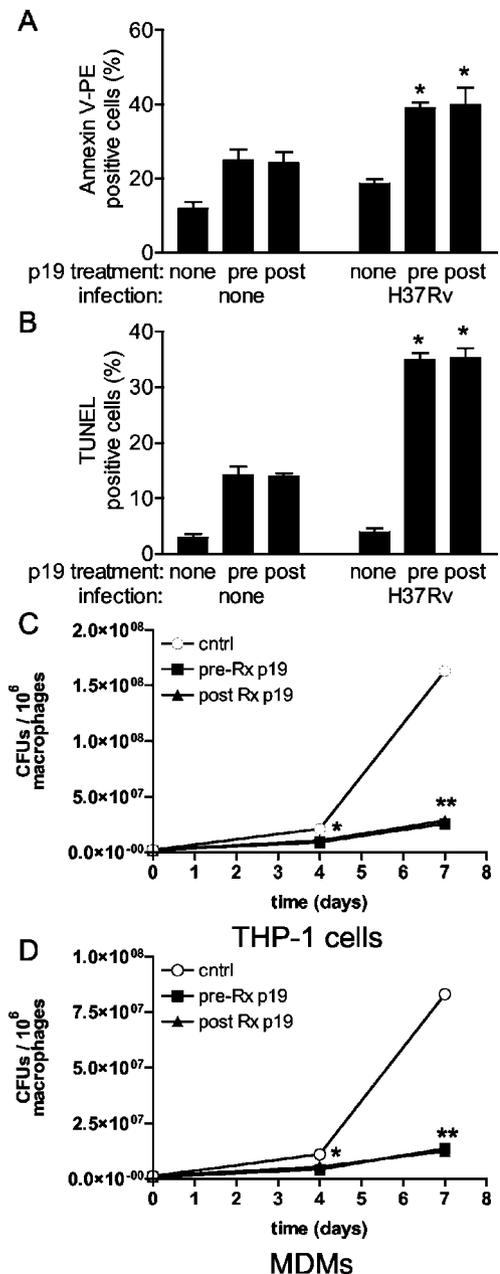
**FIGURE 3.** Apoptosis induced by p19 is TLR-2-dependent. **A**, Wild-type CHO cells or CHO cells expressing either TLR-2 or FcγR were incubated with 100 ng/ml p19 for 4 h. Cells were then washed and apoptosis was measured at 24 h by annexin V binding as described for Fig. 1. His-tagged β-galactosidase was included as a specificity control. \*,  $p < 0.05$  comparing p19 treatment of the TLR-2 receptor-transfected CHO cells with the FcγR-transfected cells. **B** and **C**, One hour before the addition of p19 (100 ng/ml), PMA-differentiated THP-1 cells (**B**) or CHO cells (**C**) were incubated with 25 μg/ml of human TLR-2-specific mAb (2392) or an isotype-matched irrelevant Ab (i/c). Incubation of THP-1 cells and CHO cells with p19 was for 2 and 4 h, respectively, followed by washing, replacement of medium, and incubation at 37°C for a total of 24 h. \*,  $p < 0.05$  comparing prior incubation with anti-TLR2 Ab to p19 treatment alone or to prior incubation with isotype-matched irrelevant control Ab. Incubation with actinomycin D (50 μg/ml for 19 h) was included as a positive control for apoptosis. The results shown are the means of three independent experiments performed in duplicate.



**FIGURE 4.** Apoptosis induced by p19 is dependent upon caspases-8 and -3 and independent of caspase-9. As a measure of apoptosis, annexin V-PE binding was measured (A) and TUNEL-positive cells were enumerated (B). One hour before the addition of p19 (50 ng/ml), PMA-differentiated THP-1 cells were treated with peptide inhibitors (75  $\mu$ M) specific for either caspase-8, caspase-3, or caspase-9 as detailed in *Materials and Methods*. Peptide Z-FA-FMK was used as a negative control. The results shown are the means  $\pm$  SD of three independent experiments performed in duplicate. \*,  $p < 0.05$  comparing p19 treatment, caspase-9 inhibitor plus p19 treatment or control inhibitor plus p19 treatment to cells with the same inhibitors in the absence of p19 treatment and comparing the caspase-8 inhibitor plus p19 treatment or the caspase-3 inhibitor plus p19 treatment to p19 treatment alone; \*\*,  $p < 0.01$  comparing actinomycin D treatment, the caspase-8 inhibitor plus actinomycin D treatment or the caspase-3 inhibitor plus actinomycin D treatment to control cells treated with identical inhibitors. \*\*\*,  $p < 0.01$  comparing the caspase-3 inhibitor plus actinomycin D treatment or the caspase-9 inhibitor plus actinomycin D to cells treated with actinomycin D alone in the TUNEL assay. \*\*\*\*,  $p < 0.02$  comparing the caspase-3 inhibitor plus actinomycin D treatment or the caspase-9 inhibitor plus actinomycin D to cells treated with actinomycin D alone in the annexin V-PE binding assay.

that cause DNA damage, granzyme B, and cathepsins, typically activate the mitochondrial pathway of PCD directly.

Recently, there has been considerable interest in understanding how microbial invasion may affect apoptosis of eukaryotic cells and whether this contributes to pathogenesis. For example, *M. tuberculosis* and other mycobacteria as well as a variety of unrelated microbial pathogens have been reported to induce apoptosis of host cells (13, 15–17, 19–22, 40–44). In some instances, apoptosis



**FIGURE 5.** Apoptosis of *M. tuberculosis*-infected cells induced by p19 results in reduced viability of intracellular bacilli. Differentiated THP-1 cells or MDMs were incubated with 50 ng/ml p19 for 2 h either alone, before, or following infection with *M. tuberculosis* H37Rv (ratio of bacteria added to cells, 50:1 for THP-1 cells or 5:1 for MDMs resulting in an infection rate of 80–90% with 1–5 bacilli/cell). Four hours after addition of bacteria, monolayers were washed and incubated for an additional 24 h. A and B, Apoptosis was measured by flow cytometric analysis of annexin V-PE-positive cells as described for Fig. 1 and by TUNEL assay, respectively.  $p < 0.01$  comparing the effect of p19 treatment whether pre- or posttreatment was different from the corresponding control. Infection alone did not bring about a significant increase in annexin V binding compared with control (uninfected, no p19) at 24 h after infection. C and D, *M. tuberculosis* CFUs were determined in THP-1 cells and MDMs, respectively, recovered on either day 4 or day 7 following infection. The results shown are the means of three independent experiments plated in triplicate. \*,  $p < 0.03$ ; \*\*,  $p < 0.01$  comparing growth in treated cells to that in untreated cells.

had been observed to occur after infection of cells with viable organisms and in other cases upon incubation of cells with subcellular bacillary factors.

With respect to *M. tuberculosis*, ongoing issues of obvious interest include identifying the factors and mechanisms leading to PCD and whether or not apoptosis of infected cells favors host resistance or progression of infection. The present study was designed to investigate the potential role of the secreted and cell wall-associated *M. tuberculosis* protein p19 as an apoptosis-inducing factor. This possibility was suggested by the recent findings that synthetic bacterial lipoproteins can signal through TLR-2 to induce apoptosis (45) and by evidence indicating that p19 may be a TLR-2 ligand (23). p19 as a candidate apoptosis-inducing factor was also suggested by the finding that pre-opsonization of *M. tuberculosis* with anti-p19 Ab abrogated the ability of bacilli to induce apoptosis upon infection of monocytes (46). However, whether secreted p19 alone could bring about PCD, the nature of the apoptotic mechanism and the components of p19 involved remain to be determined.

Using recombinant His-tagged p19 expressed in *E. coli*, our results indeed show that soluble p19 alone is an apoptosis-inducing factor. Induction of PCD by bacterial-expressed p19 was both dose- and time-dependent (Fig. 1) and was not due to either the His tag (Fig. 2A) or LPS contamination (Fig. 2, B and C). Apoptosis in response to p19 was evident within 5 min of its addition to THP-1 cells (data not shown). This suggested the likely involvement of a receptor-dependent mechanism and TLR-2 as a candidate death receptor. TLR-2 has been shown to function as a novel death receptor in that it lacks a cytoplasmic death domain typically found in classical death receptors such as Fas. In the case of TLR-2, signaling for apoptosis was found to be dependent upon MyD88, which acts as an adapter protein binding to Fas-associated death domain protein (33). To examine whether induction of PCD by p19 is TLR-2 dependent, we used anti-TLR-2 blocking Ab and CHO-TLR-2 cells. As shown in Fig. 3A, expression of TLR-2 by CHO cells was sufficient to confer sensitivity to apoptosis induction by p19 and anti-TLR-2 Ab blocked PCD in response to p19 in both THP-1 cells and CHO cells expressing TLR-2 (Fig. 3, B and C).

Whereas native p19 is both glycosylated and acylated (34, 35), the recombinant p19 expressed in *E. coli* in this study lacked both of these posttranslational modifications. Previous studies of p19 signaling through TLR-2 used purified, native protein (23, 36) and were not designed to address whether these posttranslational modifications are required for functional activity. Moreover, examination of the ability of bacterial lipoproteins to induce apoptosis through TLR-2 used synthetic lipopeptide molecules, and here again the minimal requirements for biologic activity were not addressed (33, 45). Recombinant p19 was demonstrated to be as effective as the native p19 in inducing apoptosis in THP-1 cells. Further, apoptosis induction by either recombinant or native p19 was sensitive to heat but not polymyxin B treatment (Fig. 2, B and C). Our finding that unmodified recombinant p19 is able to signal through TLR-2 to induce PCD implies that lipidation is not required for this activity.

The finding that p19-induced apoptosis appeared to involve transmembrane signaling through TLR-2 suggested that this would likely involve caspase-8 proximally and caspase-3 distally and indeed this was found to be the case (Fig. 4). Moreover, the observation that p19-induced PCD was resistant to caspase-9 inhibitor suggested a direct link between caspase-8 and caspase-3 activation independent of recruitment of the mitochondrial pathway of apoptosis. This conclusion is based upon the currently accepted model in which release of mitochondrial cytochrome *c* leads through Apaf-1 and activated caspase-9 to caspase-3 activation (39).

Important issues to address at this stage were whether p19 would also induce apoptosis of *M. tuberculosis* infected cells and if so, what would be the magnitude of this effect and the conse-

quences for the viability of intracellular bacilli. The latter in particular, would provide insight into whether PCD of infected cells would favor host resistance or promote progression of infection. As shown in Fig. 5, A and B, *M. tuberculosis* infection per se induced a minimal amount of apoptosis. Treatment of infected cells with exogenous p19 did indeed provoke apoptosis and the combined effects of infection, and p19 appeared to be additive. Whereas several studies have suggested that virulent *M. tuberculosis* may be capable of inducing an anti-apoptotic response during infection (16, 19, 20, 22, 47), these mechanisms would appear insufficient to abrogate the proapoptotic effects of p19.

In regard to the impact of apoptosis on recovery of intracellular bacilli, we observed an ~80–90% reduction in CFUs obtained from infected THP-1 cells and MDMs at 7 days following incubation with p19 (Fig. 5, C and D, respectively). The magnitude of this reduction in CFUs was comparable to that observed when *M. tuberculosis*-infected cells were induced to undergo apoptosis in response to Fas ligand (47). These results are also consistent with a previous report showing that treatment of *M. tuberculosis*-infected human monocytes with p19 resulted in bacterial killing dependent upon TLR-2 (36). Although the underlying mechanism was not identified in the latter study, based upon the results previously presented it is reasonable to speculate that it may have involved PCD. Taken together, these findings suggest that p19-induced apoptosis of infected cells may contribute to host defense by reducing the overall bacterial load. What is not clear, however, is whether the release of residual viable bacilli from apoptotic cells and their potential spread to bystander macrophages may outweigh the immediate effect of reduced bacterial viability during PCD. If indeed the net effect of apoptosis is an overall reduction in bacterial load, this would be consistent teleologically with reports showing that virulent strains of *M. tuberculosis* induce less apoptosis when compared with relatively avirulent strains (20, 22).

In summary, the results presented show that *M. tuberculosis* p19 induces apoptosis dependent upon TLR-2. Consistent with PCD being initiated through a transmembrane death receptor, apoptosis in response to p19 involved the sequential activation of caspase-8 and caspase-3 and appeared to be independent of the mitochondrial pathway of PCD. The data also suggest that the ability of p19 to induce killing of intracellular *M. tuberculosis* through TLR-2 as previously reported (36) may be related to cell apoptosis. Moreover, based upon the comparison between native p19 and recombinant p19, which lacks an acylation signal, it is reasonable to conclude that the primary structure of p19 is sufficient to confer at least some of its biologic activities, and that the lipid moiety is not required.

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