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Virulent *Mycobacterium tuberculosis* Strains Evade Apoptosis of Infected Alveolar Macrophages

Joseph Keane, Heinz G. Remold, and Hardy Kornfeld

Human alveolar macrophages (AMφ) undergo apoptosis following infection with *Mycobacterium tuberculosis* in vitro. Apoptosis of cells infected with intracellular pathogens may benefit the host by eliminating a supportive environment for bacterial growth. The present study compared AMφ apoptosis following infection by *M. tuberculosis* complex strains of differing virulence and by *Mycobacterium kansasii*. Avirulent or attenuated bacilli (*M. tuberculosis* H37Rv, *Mycobacterium bovis* bacillus Calmette-Guérin, and *M. kansasii*) induced significantly more AMφ apoptosis than virulent strains (*M. tuberculosis* H37Rv, Erdman, *M. tuberculosis* clinical isolate BMC 96.1, and *M. bovis* wild type). Increased apoptosis was not due to greater intracellular bacterial replication because virulent strains grew more rapidly in AMφ than attenuated strains despite causing less apoptosis. These findings suggest the existence of mycobacterial virulence determinants that modulate the apoptotic response of AMφ to intracellular infection and support the hypothesis that macrophage apoptosis contributes to innate host defense in tuberculosis. *The Journal of Immunology*, 2000, 164: 2016–2020.

*Mycobacterium tuberculosis* has evolved to survive and replicate inside macrophage phagosomes. It is postulated that macrophage apoptosis may contribute to host defense against this intracellular infection, analogous to apoptosis occurring in virus-infected cells. We previously reported that human alveolar macrophages (AMφ) undergo apoptosis in response to intracellular *M. tuberculosis* infection by a TNF-α-dependent mechanism (1). The virulent *M. tuberculosis* strain H37Rv was found to induce less AMφ apoptosis than the isogenic avirulent strain H37Ra. We subsequently reported that IL-10 stimulation leads to shedding of soluble TNFR2 (sTNFR2) by AMφ and that sTNFR2 can neutralize TNF bioactivity (2). TNF-α expression is critical for successful host defense of tuberculosis (3); induction of IL-10 by *M. tuberculosis* leading to inhibition of TNF-α might constitute a novel mechanism to evade host defense by virulent bacilli.

The identification of *M. tuberculosis* virulence factors is essential to understanding the pathogenesis of tuberculosis and may reveal salient components of host defense. To date, no definitive *M. tuberculosis* virulence factors have been reported and few *M. tuberculosis* virulence phenotypes in human cells have been described (4–8). We compared AMφ apoptosis in response to in vitro infection using a panel of mycobacterial strains of differing virulence. The results presented in this paper demonstrate that bacillary control of host cell apoptosis is a virulence-associated phenotype of *M. tuberculosis* and suggest that AMφ apoptosis contributes to innate immunity in tuberculosis.

**Materials and Methods**

**Alveolar macrophages**

AMφ were obtained from bronchoalveolar lavage fluid of healthy nonsmoking volunteers using standard techniques, with their informed consent under a protocol approved by the Institutional Review Board of the Boston University Medical Center. Lavage fluid was filtered through sterile gauze, centrifuged (450 × g, 10 min), and the cell pellet was suspended in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) with 10% FCS and cefotaxime 50 μg/ml. Cells were plated, and nonadherent cells were removed by washing at 24 h. Differential counts were performed on cyto-centrifuged preparations using the Leuko Stat Stain Kit (Fisher, Pittsburgh, PA). Viability of adherent AMφ was assessed by trypan blue dye exclusion.

**Mycobacteria**

A clinical strain of *M. tuberculosis* was isolated from an immunocompetent patient with pulmonary tuberculosis at the Boston Medical Center (designated BMC 96.1); *M. tuberculosis* H37Rv, H37Ra, and Erdman, as well as *Mycobacterium bovis* wild type, *M. bovis* bacillus Calmette-Guérin (BCG), and *Mycobacterium kansasii* were purchased from American Type Culture Collection (Manassas, VA). Before inoculation of AMφ, mycobacteria were dispersed by aspiration through a 25-gauge needle five times, vortexed, then sonicated (15 s, 500 W) in a bath sonicator (Laboratory Supplies, Hicksville, NY). After sonication, bacterial suspensions were allowed to stand (10 min) and the upper 500 μl were removed for use in experiments. For each experiment, the adequacy of dispersion and the multiplicity of infection (MOI) were checked by acid-fast stain of infected AMφ at 4 h. Ten high-power fields were counted to provide an equivalent MOI of 5–10 bacilli per cell for each strain examined.

**Analysis of AMφ viability**

AMφ were cultured in two-well chamber slides (Nunc, Naperville, IL) at 400,000 cells per well in 1 ml of medium (37°C, 5% CO2). Culture medium was replenished at 24 h, and at 72 h cells were infected with mycobacteria at an MOI of 5–10. After 4 h, cultures were washed to remove extracellular mycobacteria. After 5 days, culture supernatants were removed and AMφ viability was determined by staining with calcein and ethidium homodimer as previously described (1). One thousand cells counted by fluorescence microscopy on each slide were scored as live (green fluorescence) or dead (red fluorescence).

**Analysis of infected AMφ apoptosis**

AMφ in 96-well microtiter trays were infected with the different mycobacterial strains at a MOI of 5–10. After 5 days, apoptosis was measured...
Mycobacterial virulence is defined by the ability to cause progressive infection and death in animal models (10). We infected normal human AMφ with mycobacteria of differing virulence at an MOI of 5–10 bound or internalized bacilli per macrophage (determined by acid-fast staining of washed cells 4 h after infection). The high virulence strains investigated included \emph{M. tuberculosis} BMC 96.1 (a human pulmonary tuberculosis clinical isolate with minimal passage in vitro), \emph{M. tuberculosis} H37Rv, \emph{M. tuberculosis} Erdman, and \emph{M. bovis} wild type. Low virulence strains included in this analysis were \emph{M. tuberculosis} H37Ra (an isogenic attenuated strain of H37Rv), \emph{M. bovis} BCG (an isogenic avirulent strain of \emph{M. bovis}), and \emph{M. kansasii}. After 5 days in culture, AMφ viability was assessed by staining with ethidium homodimer and calcein. Consistent with our earlier studies that compared only H37Rv and H37Ra (1), all of the virulent mycobacterial strains caused significantly less AMφ cytotoxicity than the attenuated strains (Fig. 1A). As an example, BCG induced 43% ± 7% cell death (mean % dead cells ± SEM for eight experiments; \(p < 0.001\)), while infection with \emph{M. bovis} wild type was associated with no additional AMφ death over uninfected control levels of 3 ± 1%.

\textbf{AMφ apoptosis is more potently induced by attenuated than virulent mycobacteria}

To investigate relative induction of AMφ apoptosis by virulent and attenuated mycobacteria, cultures of infected cells were assayed using an apoptosis-specific ELISA for cytoplasmic histone-associated DNA fragments formed in apoptotic cells. Infection with virulent \emph{M. tuberculosis} complex strains consistently resulted in less AMφ apoptosis than infection with attenuated strains (Fig. 1B). Virulent \emph{M. tuberculosis} BMC 96.1 and H37Rv, as well as with \emph{M. bovis} wild type, failed to increase AMφ apoptosis above the baseline value for uninfected cells. In contrast, the attenuated strains H37Ra, BCG, and \emph{M. kansasii} all caused a significant increase in AMφ apoptosis over control.
AMφ apoptosis is not due to rapid intracellular mycobacterial growth

Previous experiments have reported faster intracellular growth rates by virulent mycobacteria in human monocytes and monocytic-derived macrophages (7, 8, 11). Increased AMφ cytotoxicity and apoptosis after infection by attenuated mycobacterial strains might reflect more rapid growth and accumulation of intracellular bacilli that could impair critical host cell functions. To investigate this possibility, growth in AMφ was assessed by Bactec analysis for each of the seven mycobacterial strains employed in these studies (Fig. 2). The mycobacterial content in AMφ cultures at 4 h (day 0) was compared with that at day 5 for each strain. A T-100 value (time for the inoculated Bactec vial to reach a growth index of 100) was calculated for each of the seven mycobacterial isolates, phenotypes more germane to human tuberculosis may be

Discussion

We found a consistent pattern of reduced AMφ apoptosis and cytotoxicity after infection by virulent M. tuberculosis complex bacilli as compared with attenuated or avirulent isogenic strains and M. kansasii. Virulent bacilli also consistently demonstrated faster intracellular growth than the attenuated strains despite their association with enhanced host macrophage viability. We were unable to establish a consistent relationship between the levels of TNF-α, IL-10, or sTNFR2 and the relative virulence of the infecting organism or the fate of the infected cells. While differential induction of these factors may play a role in specific cases, it appears that other mechanisms may also be involved in the modulation of AMφ apoptosis by virulent M. tuberculosis.

AMφ are the primary host cell for inhaled M. tuberculosis, which has adapted to survive and replicate within the phagosome. Apoptosis can be an effective defense strategy to limit the growth of intracellular pathogens (12). The importance of this innate defense mechanism is demonstrated by the evolutionary acquisition of apoptosis-inhibiting genes by many viruses. Our data suggest that macrophage apoptosis also plays a role in defense against M. tuberculosis. In vitro infection with M. tuberculosis induces AMφ apoptosis in a TNF-α-dependant manner (1), and apoptotic macrophages are present in pulmonary granulomas and in bronchoalveolar lavage cells from patients with tuberculosis (13, 14).

There are several mechanisms whereby macrophage apoptosis might act to limit M. tuberculosis replication in the lung. Other investigators have found that the induction of infected monocyte/macrophage apoptosis by exogenous factors, but not the induction of infected cell necrosis, limits mycobacterial growth in vitro and retains bacilli in apoptotic bodies (15, 16). In addition to depriving bacilli of an intracellular environment that facilitates growth, there is evidence that ingestion of bacilli contained in apoptotic cells by freshly added macrophages results in an augmented microbicidal effect (9). Our data presented here indicates that evasion of host AMφ apoptosis is a M. tuberculosis virulence-associated phenotype. This supports the hypothesis that apoptosis contributes to innate immunity in tuberculosis.

This is the first study to show phenotypic differences among different strains of M. tuberculosis in an in vitro assay using human AMφ. By studying the behavior of human AMφ following M. tuberculosis infection, and by employing clinical mycobacterial isolates, phenotypes more germane to human tuberculosis may be...
cial ELISA kits were used to measure TNF-α at 24 h. Identical cultures of uninfected cells served as controls. Commer-
cand M. kansasii donor. Qualitatively similar results were observed in cultures of AM infected for 5 days (data not shown).

FIGURE 3. Induction of TNF-α, IL-10, and sTNFR2 following in vitro infection of human AM with different M. tuberculosis complex strains and M. kansasii. Supernatant from cultures of infected cells was harvested with different M. tuberculosis, IL-10, and sTNFR2 following in vitro (A). Each symbol represents the values derived from a different AM (B). Supernatant from cultures of infected cells was harvested with different M. tuberculosis, IL-10, and sTNFR2 following in vitro (C). Each symbol represents the values derived from a different AM donor. Qualitatively similar results were observed in cultures of AM infected for 5 days (data not shown).

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Acknowledgments
We are grateful to Drs. Jussi Saukkonen, Michael Ieong, and Christine Reardon for assistance in bronchoscopy and to Beth Shurtleff for technical assistance.


