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Virulent *Mycobacterium tuberculosis* Strains Evade Apoptosis of Infected Alveolar Macrophages¹

Joseph Keane,²* Heinz G. Remold,[†] and Hardy Kornfeld*

Human alveolar macrophages (AM ϕ) 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 AM ϕ apoptosis following infection by *M. tuberculosis* complex strains of differing virulence and by *Mycobacterium kansasii*. Avirulent or attenuated bacilli (*M. tuberculosis* H37Ra, *Mycobacterium bovis* bacillus Calmette-Guérin, and *M. kansasii*) induced significantly more AM ϕ 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 AM ϕ than attenuated strains despite causing less apoptosis. These findings suggest the existence of mycobacterial virulence determinants that modulate the apoptotic response of AM ϕ 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.

ycobacterium 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 $(AM\phi)^3$ 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 $AM\phi$ apoptosis than the isogenic avirulent strain H37Ra. We subsequently reported that IL-10 stimulation leads to shedding of soluble TNFR2 (sTNFR2) by AM ϕ 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 AM ϕ 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 $AM\phi$ apoptosis contributes to innate immunity in tuberculosis.

Materials and Methods

Alveolar macrophages

AM ϕ 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 cytocentrifuged preparations using the Leuko Stat Stain Kit (Fisher, Pittsburgh, PA). Viability of adherent AM ϕ 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 AM ϕ , 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 μ l 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 AM ϕ 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 $AM\phi$ viability

AM ϕ 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 AM ϕ viability was determined by staining with calcein and eithidium 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 AM apoptosis

 $AM\phi$ in 96-well microtiter trays were infected with the different mycobacterial strains at a MOI of 5–10. After 5 days, apoptosis was measured

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³ Abbreviations used in this paper: AM ϕ , alveolar macrophage; BCG, bacillus Calmette-Guérin; sTNFR2, soluble TNFR2; H37Ra, *M. tuberculosis* H37Ra; H37Rv, *M. tuberculosis* H37Rv; MOI, multiplicity of infection; T-100, time to reach a Bactec growth index of 100.



FIGURE 1. Virulent *M. tuberculosis* complex strains cause less AM ϕ cell death and apoptosis than isogenic avirulent strains or the attenuated strain *M. kansasii. A*, AM ϕ were cultured on microscopy chamber slides and infected with each of seven different mycobacterial strains. Uninfected AM ϕ were used as controls. After staining with ethidium homodimer and calcein, slides were examined by epifluorescence microscopy and 1000 cells were scored as live or dead. Viability is expressed as mean % dead cells ± SEM for seven experiments. Significant differences (p < 0.05) are indicated by an asterisk. *B*, Apoptosis of infected AM ϕ as measured by histone/fragmented DNA ELISA. AM ϕ cultured in microtiter plates were infected with each of seven different strains of mycobacteria. Uninfected AM ϕ cultured in an identical manner served as controls. After 5 days, the histone and fragmented DNA content of the cells was assessed by Ag-capture ELISA. Relative apoptosis in these cultures is expressed as the mean OD ± SEM for three separate experiments. Significant differences compared with control (p < 0.05) are indicated by an asterisk.

using an Ag-capture ELISA for histone and fragmented DNA (Cell Death Detection ELISA^{PLUS}, Boehringer Mannheim, Mannheim, Germany) on cell lysate according to the manufacturer's protocol.

Assessment of mycobacterial growth

Bactec analysis of AM ϕ lysates and supernatants after bacillary infection were performed for each mycobacterial strain as previously described (9). Briefly, AM ϕ were infected with the different mycobacterial strains for 4 h or 5 days, then lysed with 0.2% SDS in PBS. SDS was neutralized by adding FCS. Cell lysate and culture supernatant from triplicate cultures were pooled and inoculated into duplicate Bactec 12B vials containing [14C]palmitic acid. Vials were incubated for 24 h at 27°C, and 14CO₂ production was determined using a Bactec 460 TB instrument that reports a growth index in arbitrary units ranging from 0 to 999. Vials were sampled every 24 h until a reading of 999 was reached. For each experiment, the time required to reach a growth index of 100 (T-100 value) was determined. Previous studies demonstrated a linear correlation between the T-100 and the log number of viable mycobacteria measured by plating and counting CFU (9). In the present study, mycobacterial growth was assessed by comparing the T-100 values 5 days after infection of AM ϕ to the initial T-100 value of the same strain 4 h after infection of AM ϕ .

Measurement of TNF- α , IL-10, and sTNFR2 release

AM ϕ were incubated in the presence or absence of mycobacteria (MOI, 5–10) in triplicate cultures. Supernatants were harvested at 24 h and 5 days and passed through a 0.22- μ m pore-size filter (Gelman Sciences, Ann Arbor, MI). The level of immunoreactive TNF- α , IL-10, and sTNFR2 was determined using commercial ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's specifications.

Statistical analysis

Cytotoxicity and apoptosis data were compared by ANOVA, and mycobacterial growth data were compared using Student's *t* test. All statistical calculations were performed with InStat software (GraphPad Software, San Diego, CA).

Results

Differential cytotoxicity of virulent and attenuated mycobacteria

Mycobacterial virulence is defined by the ability to cause progressive infection in immunocompentent humans and to cause pro-

gressive infection and death in animal models (10). We infected normal human AM ϕ 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 M. tuberculosis BMC 96.1 (a human pulmonary tuberculosis clinical isolate with minimal passage in vitro), M. tuberculosis H37Rv, M. tuberculosis Erdman, and *M. bovis* wild type. Low virulence strains included in this analysis were M. tuberculosis H37Ra (an isogenic attenuated strain of H37Rv), M. bovis BCG (an isogenic avirulent strain of M. *bovis*), and *M. kansasii*. After 5 days in culture, AM ϕ 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 AM ϕ cytotoxicity than the attenuated strains (Fig. 1A). As an example, BCG induced $43\% \pm 7\%$ cell death (mean % dead cells \pm SEM for eight experiments; p < 0.001), while infection with *M. bovis* wild type was associated with no additional $AM\phi$ death over uninfected control levels of $3 \pm 1\%$.

$AM\phi$ apoptosis is more potently induced by attenuated than virulent mycobacteria

To investigate relative induction of AM ϕ 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 *M. tuberculosis* complex strains consistently resulted in less AM ϕ apoptosis than infection with attenuated strains (Fig. 1*B*). Virulent *M. tuberculosis* BMC 96.1 and H37Rv, as well as with *M. bovis* wild type, failed to increase AM ϕ apoptosis above the baseline value for uninfected cells. In contrast, the attenuated strains H37Ra, BCG, and *M. kansasii* all caused a significant increase in AM ϕ apoptosis over control.



FIGURE 2. Growth or inhibition of different mycobacterial strains after infection of human AM ϕ . Mycobacterial growth was measured using a Bactec ¹⁴CO₂ sampler. The time to reach a Bactec growth index of 100 (T-100) decreases with increasing numbers of bacilli in any sample and is linearly correlated with log CFU determined by plating and colony counting (9). In the current study, the T-100 on day 0 reflects the number of intracellular bacilli initially introduced into the cultured AM ϕ , while the T-100 measured at day 5 reflects the number of intracellular bacilli 5 days later. The % change in T-100 over 5 days was calculated using the equation, % Δ T-100 = (T-100 day 5/T-100 day 0) × 100. A positive value represents intracellular bacterial growth over this time period, while a negative value represents a bactericidal effect. One representative of three different experiments is shown. The difference in growth rates between attenuated mycobacterial strains and virulent strains was significant (p <0.05) using an unpaired t test. A qualitatively similar result was observed in three different experiments using $AM\phi$ from different donors.

$AM\phi$ apoptosis is not due to rapid intracellular mycobacterial growth

Previous experiments have reported faster intracellular growth rates by virulent mycobacteria in human monocytes and monocyte-derived macrophages (7, 8, 11). Increased AM ϕ 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 AM ϕ was assessed by Bactec analysis for each of the seven mycobacterial strains employed in these studies (Fig. 2). The mycobacterial content in AM ϕ 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 determined for each strain and time point. The percent change in T-100 over time was calculated using the equation, $\% \Delta T$ -100 = (T-100 day 5/T-100 day 0) \times 100. The virulent mycobacterial stains that caused the least amount of cytotoxicity and apoptosis demonstrated considerable growth in the AM ϕ over 5 days. In contrast, the attenuated bacilli declined in numbers over the same time period. This indicates that increased intracellular bacillary burden is not responsible for the observed high AM ϕ apoptosis rates with these strains and suggests that host macrophage apoptosis might contribute to mycobacterial growth restriction.

Differential apoptosis induction is not related to secretion of IL-10 or TNF- α or to shedding of sTNFR2

M. tuberculosis-infected AM ϕ become primed for TNF- α -mediated cytotoxicity, and infection-induced apoptosis appears to be primarily due to autocrine or paracrine TNF- α death signals (1). We previously found that IL-10 down-regulates AM ϕ apoptosis after M. tuberculosis infection by releasing sTNFR2 that neutralizes TNF- α (2). Differences in AM ϕ apoptosis following infection by different mycobacterial strains could reflect differences in the production of TNF- α or IL-10 and/or differences in the shedding of sTNFR2 from infected cells. This question was assessed in the present study by measuring TNF- α , IL-10, and sTNFR2 in supernatants of AM ϕ 24 h and 5 days after infection with each of the seven mycobacterial strains studied. There were large variations in the response of AM ϕ from different donors, with no consistent relationship between the level of TNF- α , IL-10, or sTNFR2 and the virulence of infecting organism (Fig. 3). Similarly, no consistent relationship between cytokine or sTNFR2 levels were found in the same donor cells when infected with virulent or attenuated bacilli. The levels of TNF- α , IL-10, and sTNFR2 at day 5 were moderately increased compared with 24 h while the overall pattern of cytokine expression was similar at both time points (data not shown). These data do not exclude a role for IL-10 or sTNFR2 in regulating apoptosis of *M. tuberculosis*-infected AM ϕ , but they suggest the presence of additional mechanisms acting to modulate this response.

Discussion

We found a consistent pattern of reduced AM ϕ 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 AM ϕ apoptosis by virulent *M. tuberculosis*.

AM ϕ 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 AM ϕ apoptosis in a TNF- α -dependant manner (1), and apoptotic macrophages are present in pulmonary granulomas and in bronchoal-veolar 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 AM ϕ 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 AM ϕ . By studying the behavior of human AM ϕ following *M. tuberculosis* infection, and by employing clinical mycobacterial isolates, phenotypes more germane to human tuberculosis may be



FIGURE 3. Induction of TNF- α , IL-10, and sTNFR2 following in vitro infection of human AM ϕ with different *M. tuberculosis* complex strains and *M. kansasii*. Supernatant from cultures of infected cells was harvested at 24 h. Identical cultures of uninfected cells served as controls. Commercial ELISA kits were used to measure TNF- α (*A*), sTNFR2 (*B*), and IL-10 (*C*). Each symbol represents the values derived from a different AM ϕ donor. Qualitatively similar results were observed in cultures of AM ϕ infected for 5 days (data not shown).

described. Mycobacterial growth rates in a variety of human cells have been investigated, and it has been reported that virulent strains replicate faster than avirulent strains (4-8, 11). The basis for this phenomenon has not been established, but our findings suggest that differential induction of infected macrophage apoptosis may be an important factor. Attenuated bacilli caused more AM ϕ cytotoxicity than virulent strains in our experiments, yet the growth of the attenuated strains was restricted. This is consistent with previous reports that the use of exogenous agents such as H₂O₂ to cause apoptosis of mycobacteria-infected macrophages results in mycobacterial death (15). Our studies are unique in that apoptosis occurred as a direct result of mycobacterial infection, better reflecting events occurring naturally in tuberculosis. The capacity of virulent mycobacteria to modulate AM ϕ apoptosis can reasonably be related to the preservation of a supportive intracellular environment for bacterial growth. By inhibiting host macrophage apoptosis, the mycobacteria also avoid being packaged in apoptotic bodies that are subject to secondary phagocytosis by newly recruited mononuclear cells. It is postulated that uptake of bacilli packaged in this way leads to more effective intracellular microbicidal processing (9).

TNF- α and IL-10 have central roles in the innate response to *M*. tuberculosis infection (3, 17), and we described the influence of these cytokines on AM ϕ apoptosis after *M. tuberculosis* infection (2). We found that TNF- α and IL-10 responses of primary human AM ϕ to *M. tuberculosis* infection do not correlate with microbial virulence, suggesting that additional mechanisms also are involved in the modulation of infected AM ϕ apoptosis. The identification of contrasting apoptosis-induction phenotypes by the isogenic pairs H37Ra and H37Rv, as well as BCG and *M. bovis* wild type, may offer a means for identifying the microbial genetic basis for this difference. Analysis of apoptosis responses by murine macrophage cell lines suggests that host genetic factors may also contribute to the regulation of cell fate in tuberculosis (18). While we observed significant variability in cytokine production by $AM\phi$ from different human donors, the pattern of apoptosis responses has been very consistent in our experience.

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