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Mycobacterium tuberculosis bacilli are intracellular organisms that reside in phagosomes of alveolar macrophages (AMs). To determine the in vivo role of AM depletion in host defense against M. tuberculosis infection, mice with pulmonary tuberculosis induced by intranasal administration of virulent M. tuberculosis were treated intranasally with either liposome-encapsulated dichloromethylene diphosphonate (AM\textsuperscript{+} mice), liposomes, or saline (AM\textsuperscript{-} mice). AM\textsuperscript{-} mice were completely protected against lethality, which was associated with a reduced outgrowth of mycobacteria in lungs and liver, and a polarized production of type 1 cytokines in lung tissue, and by splenocytes stimulated ex vivo. AM\textsuperscript{-} mice displayed deficient granuloma formation, but were more capable of attraction and activation of T cells into the lung and had increased numbers of pulmonary polymorphonuclear cells. These data demonstrate that depletion of AMs is protective during pulmonary tuberculosis. The Journal of Immunology, 2001, 166: 4604–4611.

Mycobacterium tuberculosis is responsible for much morbidity and mortality worldwide (1). The increasing incidence of antibiotic resistance, together with synergism between HIV and tuberculosis, has heightened our interest in the important infectious disease and in mechanisms contributing to antimicrobial host defense.

Mycobacteria are intracellular pathogens that are taken up by host alveolar macrophages (AMs), in which they either are killed or survive. Surviving bacilli start to proliferate and are released, leading to infection of additional host cells. Apoptosis of AMs could be an effective weapon to kill or inhibit the growth of intracellular mycobacteria. Several findings suggest that AM apoptosis plays an important role in tuberculosis. Infection of human AMs with M. tuberculosis has been shown to induce apoptosis in vitro (2). Furthermore, extensive apoptosis (50–70%) was found within tuberculous granulomas in lungs of tuberculosis patients (2), and a significant increase in the number of apoptotic AMs was observed in bronchoalveolar lavage fluid (BALF) from patients with active pulmonary tuberculosis (3, 4). Despite these observations, it is not clear which role AM apoptosis plays in the pathobiology of this disease and whether it increases or decreases the mycobacterial load in vivo. In vitro studies suggest that apoptosis may be a macrophage defense mechanism to infection by mycobacteria. Indeed, apoptosis of human monocytes limited the growth of Mycobacterium avium (5), Mycobacterium bovis bacillus Calmette-Guerin (6), and M. tuberculosis (7). However, in vitro studies are not adequate to determine the net effect of AM depletion on the host response to tuberculosis. AMs have important phagocytic and immune functions that could be disturbed by the apoptotic process. Clearance of microorganisms that reach the alveolar space relies partly on phagocytic AMs. Furthermore, macrophages present mycobacterial Ags to CD4\textsuperscript{+} T lymphocytes that are central in the acquired resistance to M. tuberculosis. Macrophages are a significant source of type 1 cytokines during mycobacterial infection (8), which are known to be important for the development of protective immunity (9). In addition, AMs produce IFN-γ in response to M. tuberculosis (10), which is a pivotal mediator in host resistance to tuberculosis (11, 12). Finally, mononuclear cells are involved in the formation of granulomas, which are critical in restricting mycobacterial growth and dissemination (13). Hence, theoretically AM depletion could have beneficial and detrimental effects during tuberculosis in vivo.

In the present study, we determined the role of AM depletion in M. tuberculosis infection in mice, using the well-validated method of intrapulmonary delivery of liposome-encapsulated dichloromethylene diphosphonate (clodronic-acid disodium salt tetrahydrate, CL\textsubscript{2} MBP). Intratracheal administration of liposome-encapsulated CL\textsubscript{2} MBP selectively depletes AMs (14) by apoptosis (15, 16) without damaging other cell types in the lung (17). In this work, we present the first evidence that AM depletion in vivo leads to improved clearance of M. tuberculosis bacilli.

Materials and Methods

Mice

Pathogen-free 6-wk-old female BALB/c mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands) and were maintained in biosafety level 3 facilities. The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.
Experimental infection
A virulent laboratory strain of \textit{M. tuberculosis} H37Rv was grown in liquid Dubos medium containing 0.1% Tween 80 for 4 days. A replicate culture was incubated at 37°C, harvested at mid-log phase, and stored in aliquots at -70°C. For each experiment, a vial was thawed and washed twice with sterile 0.9% NaCl. Mice were anesthetized by inhalation with isoflurane (Abbott Laboratories, Kent, U.K.) and injected with 1 \times 10^7 live bacilli in 50 \mu l saline, as determined by viable counts on 7H11 Middlebrook agar plates. Bacterial administration was performed intranasally (i.n.), as described previously (18–20). Groups of eight mice per time point were sacrificed 2 or 5 wk postinfection, and lungs and one lobus of the liver were removed aseptically. Organs were homogenized with a tissue homogenizer (Biospec Products, Bartlesville, OK) in 5 vol of sterile 0.9% NaCl, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21-day incubation at 37°C. Numbers of CFUs are provided as total in the lungs or as total per gram liver. For cytokine measurements, lungs homogenates were diluted 1/1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl\_2, 1% Triton X-100, 100 \mu g/ml pepstatin A, leupeptin, and aproptin), and incubated on ice for 30 min. Supernatants were sterilized using a 0.22-\mu m filter (Corning, Corning, NY) and frozen at -20°C until assays were performed.

In vivo AM depletion
CL\_2 MBP was a gift from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing CL\_2 MBP was done as described previously (17). For assessment of AM depletion, five uninfected mice per group were i.n. inoculated with 100 \mu l of 0.9% NaCl, PBS liposomes, or CL\_2 MBP liposomes. Two days later, AMs were quantified in the BALF. For the tuberculosis experiments, 100 \mu l saline, PBS liposomes, or CL\_2 MBP liposomes were instilled 2 days before and 6, 14, and 25 days after \textit{M. tuberculosis} challenge.

Detection of apoptotic cells
To confirm apoptotic cell death induced by CL\_2 MBP liposomes, a cleavage of poly(ADP-ribose) polymerase (PARP) was determined, as described previously (21). Briefly, 7 h after i.n. instillation of CL\_2 MBP liposomes, tissue Tek OTC compound (Miles Scientific, Naperville, IL) was instilled intratracheally into lungs, which were then snap frozen and stored at -70°C. Cryostat sections (7 \mu m) of frozen lungs were fixed in cold acetone for 10 min, incubated with 0.3% H\_2O\_2 in methanol for 15 min, blocked for nonspecific Ig binding by incubation for 30 min with a 1/10 dilution of normal goat serum, and incubated overnight with rabbit anti-PARP cleavage site (214/215)-specific Ab (Biosource International, Camarillo, CA; 5 \mu g/ml). This was followed by a 30-min incubation with poly-HP-RP goat anti-rabbit IgG (Immunovision, Springdale, AZ). The peroxidase activity was revealed by adding AEC substrate (3-amino-9-ethyl-carbazole; Sigma, Buchs, Switzerland) and H\_2O\_2. Sections were counterstained with hematoxylin. Negative controls were established by adding nonspecific isotype controls as primary Abs.

Assessment of in vitro effect of CL\_2 MDP liposomes on \textit{M. tuberculosis}
A total of 2.5 \times 10^7 CFUs was incubated in ocultiplate in 96-well bottom culture plates in the presence of Lowenstein-Jensen medium (Becton Dickinson, Franklin Lakes, NJ) with either 0.9% NaCl, PBS liposomes, or CL\_2 MBP liposomes. After 48-h incubation at 37°C in 5% CO\_2, colonies were counted.

Lung lavage
Bronchoalveolar lavage was performed to obtain intraalveolar cells. Briefly, mice were anesthetized, and the trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). The lungs were then lavaged with two 0.5-ml aliquots of sterile 0.9% NaCl. A total of 0.9–1 ml of lavage fluid was retrieved per mouse, and total leukocyte count was determined using a hemacytometer and TURK’s solution (Merck, Gibbstown, NJ). BALFs from infected mice were fixed with 2% paraformaldehyde. The number of AMs, polymorphonuclear cells (PMNs), and lymphocytes were calculated from these totals, using cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGaw Park, IL).

Histological analyses
The left lungs were removed 2 or 5 wk after inoculation with \textit{M. tuberculosis} and fixed in 4% paraformaldehyde in PBS for 24 h. One lobus of the liver of noninfected mice was removed 2 days after CL\_2 MBP liposome treatment. After embedding in paraffin, 4-\mu m-thick sections were stained with eosin hematoxylin-eosin or the Ziehl-Neelsen (ZN) stain for acid fast bacilli. All slides were coded and semiquantitatively scored for the total area of inflammation (percentage of surface of the slide) and granuloma format by a pathologist.

FACS analysis
Lung cells from mice 2 and 5 wk postinfection (eight mice per group) were analyzed by FACS (Becton Dickinson). Pulmonary cell suspension was obtained using an automated disaggregation device (Medimachine System, Dako, Glostrup, Denmark) and resuspended in medium (RPMI 1640 (Bio-Whittaker, Belgium), 10% FCS, 1% anti-biocytin-antimycolic (Life Technologies, Rockville, MD)). Cells from two mice per group were pooled for each time point (yielding four samples for FACS analysis per group) and were brought to a concentration of 4 \times 10^6 cells/ml FACS buffer (PBS supplement with 0.5% BSA, 0.01% NaN\_3, and 100 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using directly labeled Abs against CD3 (anti-CD3 PE), CD4 (anti-CD4 CyChrome), CD8 (anti-CD8 FITC), anti-CD8 PerCP), CD25 (anti-CD25 FITC), CD69 (anti-CD69 FITC), and Gr-1 (anti-Gr-1 FITC). All Abs were used in concentrations recommended by the manufacturer (PharMingen, San Diego, CA). To correct for specific staining, an appropriate control Ab (rat IgG2; PharMingen) was used. Cells were fixed with 2% paraformaldehyde, T cells were analyzed by gating the CD3\+_ population, and granulocytes by gating the forward and side angle scatter-gated PMN population. The number of positive cells was obtained by setting a quadrant marker for nonspecific staining.

Splenocyte stimulation
Single cell suspensions were obtained by crushing spleens through a 40-\mu m cell strainer (Becton Dickinson). Erythrocytes were lysed with cold isotonic NH\_4Cl solution (155 mM NH\_4Cl, 10 mM KHCO\_3, 100 mM EDTA, pH 7.4), and the remaining cells were washed twice. Splenocytes were suspended in medium (RPMI 1640 (Bio-Whittaker), 10% FCS, 1% anti-biocytin-antimycolic (Life Technologies)), seeded in 96-well bottom-tom culture plates at a cell density of 5 \times 10^5 cells in triplicate, and stimulated with 20 \mu g/ml tuberculin-purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark). Supernatants were harvested after a 48-h incubation at 37°C in 5% CO\_2, and cytokine levels were analyzed by ELISA.

Splenocyte proliferation assay
Proliferation of splenocytes was measured by the MTT assay, which measures reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formasan in mitochondria of viable cells (22). Splenocytes were seeded in triplicate at a density of 5 \times 10^5 cells/well in flat-bottom 96-well plates and stimulated with 20 \mu g/ml PPD. After 42 h at 37°C in 5% CO\_2, cells were incubated with 5 mg/ml MTT (Sigma, St. Louis, MO) in PBS (pH 7.2) for an additional 6 h. Supernatants were decanted, and the formazan precipitates were solubilized by the addition of 0.04 N HCl in isopropanol and placed on a plate shaker for 10 min, after which cells were dissolved in 2% paraformaldehyde. Cell proliferation was quantified using an ELISA reader at 570 nm. The absorbance of the untreated cultures was set at 100%.

Cytokine measurements
Cytokines were measured in lung homogenates and spleen cell supernatants by specific ELISAs using matched Ab pairs according to the manufacturer’s instructions: IFN-\gamma, IL-2, IL-4 (R&D Systems, Minneapolis, MN), and IL-10 (PharMingen).

Statistical analysis
All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests. For comparison of survival curves, Kaplan-Meier analysis with a log rank test was used. Values of \(p \leq 0.05 \) were considered statistically significant.

Results
Effects of AM depletion on the course of infection
Intranasal administration of liposome-encapsulated CL\_2 MBP resulted in >70% AM depletion in BALF of uninfected mice after 2
CL2 MBP liposomes or CL2 MBP liposomes given by aerosol to previous reports on the capacity of intratracheally administered large areas of degenerated macrophages with cell debris and deplete AMs (23, 24). Induction of AM apoptosis by CL2 MBP mortality after 5 mo. In sharp contrast, all AM mice decreased extensively from day 35 onward, resulting in 90% 25 days after induction of tuberculosis (AM mice). Survival and bacteria deposited in the lungs and liver were analyzed to determine resistance to tuberculosis. As shown in Fig. 2, survival in AM+ (saline) mice decreased extensively from day 35 onward, resulting in 90% mortality after 5 mo. In sharp contrast, all AM mice controlled 2 wk postinfection (Fig. 3a). At 5 wk postinfection, the number of organisms in the liver of AM mice was 3.6 times lower than that in AM+ (saline) mice (p = 0.021, Fig. 3a) and 7.1-fold less than of AM+ (liposomes) mice (p = 0.035).

To give more clarity on the residing place of mycobacteria in AM+ animals, lavage fluids of these mice were analyzed with a Ziehl-Neelsen (ZN) stain for acid fast bacilli. In BALFs of AM+ mice, mycobacteria were present within the cytoplasm of 39 ± 4% (2 wk postinfection) and 47 ± 10% (5 wk postinfection) of the macrophages and in few PMNs. In the BALFs of AM− mice, we found extracellular mycobacteria, but mycobacteria were especially present in cell debris. At 2 wk postinfection, the mycobacterial load in the liver of AM− mice was increased in comparison with AM+ (saline) mice (p = 0.02) and AM+ (liposomes) mice (p = 0.004, Fig. 3b). At 5 wk postinfection, the number of organisms in the liver of AM− mice was 3.6 times lower than that in AM+ (saline) mice (p = 0.011, Fig. 3b) and 2.7 times lower than in AM+ (liposomes) (not significantly different). Bacterial counts in lungs and liver of the control groups treated with either saline or liposomes were not significantly different at either time point.

To exclude the possibility that liposome-encapsulated CL2 MBP had a direct effect on mycobacteria, M. tuberculosis was incubated in vitro in the presence or absence of this agent for 2 days. Bacterial counting demonstrated no direct antimycobacterial effect of liposome-encapsulated CL2 MBP (data not shown).

Together these findings suggest that AM depletion by apoptosis can play an important role in controlling M. tuberculosis infection.

**Histology**

Two weeks after M. tuberculosis inoculation, lungs of AM+ (saline/liposomes) mice exhibited more or less well-defined granulomas comprising a majority of epithelioid and foamy cells and a small number of lymphocytes throughout the parenchyma. By contrast, BALB/c mice (n = 10 per group) were i.n. administered with saline, liposomes, or CL2 MBP liposomes prior to and after bacterial challenge of 1 × 10⁷ M. tuberculosis H37Rv. *p < 0.05 AM+ mice vs AM− (saline) mice; †, p < 0.05 AM+ mice vs AM− (liposomes) mice; ‡, p < 0.05 AM+ (saline) mice vs AM− (liposomes) mice.

Because of the impressive differences in survival, we determined whether differences existed in mycobacterial load during earlier phases of the infection. The number of bacteria deposited in the lungs and liver was determined 1 day after infection. Bacterial counts in the lungs were comparable with the numbers of bacteria that were given intranasally and did not differ between the groups. The numbers of M. tuberculosis CFUs recovered from lungs were not significantly different between AM+ (saline/liposomes) and AM− mice 2 wk postinfection (Fig. 3a). At 5 wk postinfection, significant differences in tissue content of M. tuberculosis bacilli were observed between AM+ and AM− mice. The lungs of AM− mice contained 9.7-fold less viable mycobacteria than those of AM+ (saline) animals (p = 0.021, Fig. 3a) and 7.1-fold less than of AM+ (liposomes) mice (p = 0.035).

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of granulocytes with prominent perivascular lymphocytic infiltrates. Well-defined granulomas were not present (Fig. 4 b). The percentage of inflamed parenchyma was similar in all groups (AM+ (saline) mice, 21.25 ± 3.5%; AM+ (liposomes) mice, 16.9 ± 3.5%; AM− mice, 18.8 ± 2.9%). After 5 wk, the inflammatory infiltrates in lungs of all mice became more diffuse and intense with a cellular composition comparable in AM+ (saline/liposomes) and AM− mice. However, the percentage of inflamed parenchyma was less in AM− mice (45 ± 4.8%) than in AM+ mice (saline, 61.9 ± 4.9%; liposomes, 62.9 ± 7.1%) (Fig. 4, c and d).

Cell subsets

CD4+ T cells have an established role in protective immunity against M. tuberculosis infection (25–27), and must be stimulated with specific ligands on the surface of APCs. To study whether AMs are important for the induction of CD4+ T cell-mediated immunity, we investigated the phenotypes of immune cells in total lungs by FACS analysis. As shown in Table I, the percentages of CD4+ T cells did not differ between AM+ and AM− mice 5 wk postinfection and were slightly reduced in AM− mice 2 wk postinfection. Two weeks after infection, CD4+ lymphocytes of AM− mice were demonstrated to be more activated than CD4+ lymphocytes of AM+ mice, as assessed by the activation markers CD69 and CD25.

Besides CD4+ T cells, CD8+ T cells have also been suggested to participate in host defense against mycobacterial infections (25). The percentage of CD8+ T cells in lung homogenates was not changed in AM− mice as compared with AM+ (saline/liposomes) mice 5 wk postinfection and slightly increased 2 wk postinfection. The expression of the activation marker CD69 on these cells was slightly increased. CD25 expression on CD8+ cells could not be detected. The absolute number of PMNs was higher in AM− mice than in AM+ control animals 2 wk postinfection. At 5 wk, numbers of total leukocytes were decreased in AM− mice compared with AM+ (saline/liposomes) mice, probably reflecting disease severity.

To obtain more insight into the leukocyte influx into the alveolar compartment, lungs were lavaged, cells were counted, and cyto- spin preparations were stained with eosin hematoxylin-eosin (Table II). Two weeks postinfection, the number of leukocytes was higher in AM− mice than in AM+ (saline/liposomes) mice. In line with the numbers of leukocytes in total lungs at 5 wk postinfection, cell numbers in BALFs were lower in AM− mice than in AM+ (saline/liposomes) mice. As could be expected, CL2MBP liposome-treated animals had 2 times less AMs in their BALFs than AM+ (saline/liposomes) mice. The amount of PMNs and lymphocytes in AM− mice was however increased 2 wk postinfection in comparison with AM+ (saline/liposomes) mice. As a consequence of lower leukocyte numbers in AM− mice 5 wk postinfection, numbers of PMNs and lymphocytes were decreased in this group compared with AM+ (saline/liposomes) mice.

Cytokine expression patterns in lung

Since development of early Th1 cellular immunity is essential for the elimination of M. tuberculosis (9), we investigated whether the improved outcome of tuberculosis seen in the AM− mice was associated with a shift in cytokine production early in the infection. We therefore measured the concentrations of Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-10) cytokines in the lung. As shown in Fig. 5, all cytokines were reduced in AM− mice compared with AM+ (saline/liposomes) mice 2 wk postinfection. Importantly, when compared with AM+ (saline/liposomes) mice, Th2 cytokine concentrations were relatively more reduced than the levels of Th1 cytokines in AM− mice. As a consequence, a more profound Th1 response was found in lungs of AM− mice.

Cytokine and proliferative response of ex vivo stimulated spleen cells

The ability of spleen cells, harvested 2 wk postinfection with M. tuberculosis to produce cytokines ex vivo upon stimulation with PPD, was investigated as another measure of Th1 vs Th2 response. Spleen cells from AM− mice secreted 3.5-fold higher levels of IFN-γ than splenocytes from AM+ (saline) mice and 2-fold higher levels than splenocytes from AM+ (liposomes) mice (Fig. 6). IL-4 was not detectable in supernatants of PPD-stimulated splenocytes in all groups. When stimulated with coated anti-CD3 and anti-CD28 Abs, splenocytes from AM− mice secreted higher levels of IFN-γ and significantly lower levels of IL-4 compared with AM+ (saline) animals. In addition, the proliferation responses of splenocytes to PPD were estimated using the MTT incorporation assay. We found that splenocytes from AM− mice induced the strongest proliferative response to PPD, although not statistically significant (AM+ (saline) mice, 155 ± 17%; AM+ (liposomes) mice, 158 ± 6%; and AM− mice, 195 ± 31%).
Discussion

AMs may have a dual role during infection with *M. tuberculosis*. On the one hand, AMs have several tools to combat intracellular pathogens, such as the production of IFN-γ and toxic effector molecules (reactive oxygen intermediates and reactive nitrogen intermediates), and the deprivation of the intracellular iron availability. On the other hand, mycobacteria may in part rely on the intracellular environment of AMs for their multiplication. We demonstrate in this study that depletion of AMs in vivo improves the outcome of pulmonary tuberculosis, as indicated by a total protection against lethality and an attenuated outgrowth of mycobacteria in lungs. These results suggest that AMs facilitate the growth of *M. tuberculosis* in the pulmonary compartment, and that AM apoptosis may be part of the host defense mechanisms during tuberculosis. Interestingly, AMs do seem to have a significant role in the initial capturing of mycobacteria, as indicated by the observation that 2 wk postinfection AM⁺ mice had more mycobacteria in their livers.

Table 1. Effect of AM depletion on cell subsets in total lungs during tuberculosis

<table>
<thead>
<tr>
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<th>2 wk postinfection</th>
<th>5 wk postinfection</th>
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<tbody>
<tr>
<td></td>
<td>Cells (×10⁵/ml)</td>
<td>CD4⁺</td>
</tr>
<tr>
<td>AM⁺ (saline)</td>
<td>356 ± 63.9</td>
<td>69.9 ± 1</td>
</tr>
<tr>
<td>AM⁺ (liposomes)</td>
<td>265.7 ± 50.4</td>
<td>65.6 ± 1.2*</td>
</tr>
<tr>
<td>AM⁻</td>
<td>444.5 ± 111.6</td>
<td>63 ± 5.3*</td>
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<th>5 wk postinfection</th>
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<tr>
<td></td>
<td>AM⁺ (saline)</td>
<td>225 ± 26</td>
</tr>
<tr>
<td>AM⁺ (liposomes)</td>
<td>207.8 ± 11.5</td>
<td>52 ± 1.3</td>
</tr>
<tr>
<td>AM⁻</td>
<td>136.5 ± 15.1*</td>
<td>53.9 ± 1</td>
</tr>
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</table>

*Cell subsets in the lungs of mice infected with *M. tuberculosis*, 2 and 5 wk postinfection. FACS analysis was performed on pooled cells from two mice for each analysis from a total of eight mice per group for each time point as described in Materials and Methods. FACS results are expressed as the percent of CD4⁺, CD8⁺, CD25⁺, and CD69⁺ within the CD3⁺ population or the percent of Gr-1⁺ within the PMN population.

*, p < 0.05 AM⁺ mice vs AM⁺ (saline) mice; †, p < 0.05 AM⁺ mice vs AM⁺ (liposomes) mice; and ‡, p < 0.05 AM⁺ (saline) vs AM⁺ mice (liposomes).
Host cell apoptosis has already been demonstrated to be a defense strategy to limit the growth of viruses, which like mycobacteria live intracellularly (28–30). The fact that AM apoptosis might contribute to host defense is further supported by observations of an inverse relationship between apoptosis and virulence, i.e., the virulent M. tuberculosis strain H37Rv induced less apoptosis upon human AM infection than the attenuated H37Ra strain (2). Hence, mycobacteria seem to have developed ways to modulate the protective apoptotic process of AMs, and pathogen-induced suppression of the host cell-death pathway may serve to evade host defenses that can act to limit the infection. It should be noted that the role of AMs in respiratory infections by extracellularly growing pathogens is opposite. Indeed, induction of AM apoptosis during Klebsiella pneumonia impaired host defense mechanisms (31).

The most straightforward interpretation of the improved tuberculosis outcome in AM− mice is that AM depletion reduces the viability of M. tuberculosis because the environment for intracellular replication and hiding is destroyed (32). Furthermore, apoptotic bodies maintain their plasma membrane integrity so that bacilli are contained from the extracellular environment and can be engulfed by newly recruited AMs (5). A further explanation for the protection observed with AM depletion may be that the early immune response was dominated by a Th1-type profile that is essential for resistance to mycobacteria (9). The predominance of Th1-type cytokines in AM− mice existed both in lung tissue, in which especially the concentrations of Th2 cytokines were decreased, and in supernatants of PPD-stimulated splenocytes. Wang et al. (8) recently reported that lung macrophages harvested during mycobacterial infection release significant amounts of type 1 cytokines. In line with these observations, we found lower levels of IFN-γ and IL-2 in lung homogenates 2 wk postinfection. However, the net in vivo effect of AM depletion was a relative type 1 dominance in the lung. It is unlikely that this type 1 shift was the consequence of a milder inflammatory response in AM− mice, which, if anything, showed slightly more evidence of inflammation (Tables I and II). It is conceivable that the depletion of AMs was involved in this shift. AMs are typical macrophage populations, which are known to induce differentiation of naive T cells into Th2 type cells, and to exert Th2-associated effector functions (33, 34).

### Table II. Effect of AM depletion on cellular composition on BALFs during tuberculosis

<table>
<thead>
<tr>
<th></th>
<th>Cells (×10⁴/ml)</th>
<th>AM (×10⁴/ml)</th>
<th>PMN (×10⁴/ml)</th>
<th>Lymphocytes (×10⁴/ml)</th>
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<tbody>
<tr>
<td>2 wk postinfection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM+ (saline)</td>
<td>79 ± 7.2</td>
<td>32.9 ± 5.7</td>
<td>28.8 ± 6.1</td>
<td>17.2 ± 1</td>
</tr>
<tr>
<td>AM+ (liposomes)</td>
<td>63.8 ± 7.5</td>
<td>31.8 ± 1.7</td>
<td>19.5 ± 6.4</td>
<td>14 ± 3.4</td>
</tr>
<tr>
<td>AM−</td>
<td>111 ± 7.3*†</td>
<td>16.3 ± 2.9*†</td>
<td>53.6 ± 4.2*†</td>
<td>41 ± 5*†</td>
</tr>
<tr>
<td>5 wk postinfection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM+ (saline)</td>
<td>308 ± 17.0</td>
<td>44 ± 3.4</td>
<td>182 ± 14</td>
<td>82.8 ± 16.7</td>
</tr>
<tr>
<td>AM+ (liposomes)</td>
<td>196 ± 27.6†</td>
<td>27.2 ± 6.1†</td>
<td>120 ± 23.4</td>
<td>54.6 ± 10.5</td>
</tr>
<tr>
<td>AM−</td>
<td>166 ± 16.7*</td>
<td>14.0 ± 1.5*</td>
<td>132.5 ± 13.9*</td>
<td>43 ± 5.5*</td>
</tr>
</tbody>
</table>

* Leukocytes in BALFs of mice infected with M. tuberculosis, 2 and 5 wk postinfection. Cells from four mice were counted and stained with hematoxylin and eosin. †, p < 0.05 AM− mice vs AM+ (saline) mice; †, p < 0.05 AM− mice vs AM+ (liposomes) mice; and †, p < 0.05 AM− (saline) vs AM+ mice (liposomes).

**FIGURE 5.** The effect of AM depletion on M. tuberculosis-mediated induction of type 1 cytokines (a) and type 2 cytokines (b) in lungs of AM+ (saline) mice (○), AM+ (liposomes) mice (■), and AM− mice (■) 2 wk postinfection. The data represent the mean and SEM of eight mice. *, p < 0.05 AM− mice vs AM+ (saline) mice; †, p < 0.05 AM− mice vs AM+ (liposomes) mice.
AMs can reduce the phagocytic and migratory behavior of AMs and may therefore influence host defense against M. tuberculosis. In accordance, animals treated i.n. with liposomes only (i.e., without CL₂ MBP), displayed an enhanced survival and a slight (not significant) reduction in M. tuberculosis CFU in lungs and liver compared with AM⁺ (saline) mice. Since we sought to determine the role of AMs in pulmonary tuberculosis, control mice should have normal, nonsuppressed AMs, and in this way we ensure that all functions of the macrophages that have ingested this compound are abrogated. AMs that phagocytosed the liposomes alone are expected to have some functional disabilities. Nonetheless, AM⁺ liposome-treated mice differed significantly from AM⁻ mice with respect to all responses measured.

This study is the first to show that AM depletion in vivo is protective in M. tuberculosis infection and that it is associated with an enhanced Th1-mediated immune response. AM apoptosis as observed in patients with tuberculosis could therefore be an important antimycobacterial defense process. The present results not only provide new insights into possible macrophage antimicrobial defense mechanisms, but also reveal potentially new therapeutic strategies to manage intracellular bacterial diseases.

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References


