Macrophages play a dual role during pulmonary tuberculosis in mice

Published in:
The Journal of Infectious Diseases

DOI:
10.1086/426395

Citation for published version (APA):
Macrophages Play a Dual Role during Pulmonary Tuberculosis in Mice

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Lungs are subjected to constant environmental exposure to microorganisms and particles. As a consequence, infections arise more frequently in the lungs than in any other organ, which implies that an appropriate pulmonary defense mechanism is essential. An important causative agent of pulmonary lung infection is the intracellular pathogen Mycobacterium tuberculosis, which causes more deaths annually than any other single infectious agent [1]. Because M. tuberculosis replicates predominantly in macrophages, its pathogenicity is critically dependent on the outcome of the interaction between the macrophage and the bacterium. There is evidence that macrophages can destroy M. tuberculosis by different mechanisms. They can deliver lysosomal enzymes to phagosomes, produce toxic effector molecules, limit the availability of iron to mycobacteria, and undergo apoptosis. Paradoxically, macrophages are also the main host cells of M. tuberculosis. Inside the macrophage, tubercle bacilli can replicate and are protected from extracellular host defense mechanisms such as complement and specific antibodies.

In accordance with the possibility that macrophages may facilitate mycobacterial growth in lungs, we recently found that depletion of ~70% of alveolar macrophages before M. tuberculosis infection completely protected mice against lethality and led to attenuated mycobacterial outgrowth [2]. This led us to hypothesize that macrophages play a dual role in M. tuberculosis infection. It can be anticipated that the activation state of macrophages is important in determining whether these cells facilitate or inhibit the growth of mycobacteria. Indeed, macrophages that have been activated in vitro by cytokine treatment have been demonstrated to inhibit the growth of mycobacteria [3], although other researchers have reported that this might be an artefact [4]. To study the in vivo correlation of macrophage activation with antimicrobial functions, macrophage-activating and -inactivating cytokines have often been used [5].

Because the exact timing, combination, and sequence of the signals needed for sufficient activation of mac-
rophones have not been identified, we used a different approach to
determine the influence of the activation state of macrophages on their role in tuberculosis (TB) in vivo. We depleted mice of activated pulmonary macrophages by inducing apoptosis. For this purpose, we used transgenic mice expressing human (h) FcyRI (CD64), which is up-regulated on activation [6, 7], and treated them with anti–hFcyRI monoclonal antibody (MAb) conjugated to ricin-A, after M. tuberculosis infection. In a previous study, Thepen et al. [8] demonstrated that this immunotoxin (IT) targeted only activated inflammatory macrophages, leaving inactivated, low FcyRI–expressing cells unaffected. Additionally, it has been demonstrated that this IT depletes activated macrophages and leukemia cells by inducing apoptosis [8, 9]. For comparison, we also determined the role of nonselective depletion of macrophages after M. tuberculosis infection, using the well-validated method of intrapulmonary delivery of liposome-encapsulated dichloromethylene bisphosphonate (Cl2MBP). Intratracheal administration of liposome-encapsulated Cl2MBP selectively depletes alveolar macrophages [10] by apoptosis [11, 12] without damaging other cell types in the lung [13]. Considering this, we studied the in vivo role of activated macrophages in the host defense against M. tuberculosis and determined whether the mycobacterial effects of the nonselective depletion of macrophages by apoptosis are distinct from those of induction of apoptosis in activated macrophages.

MATERIALS AND METHODS

Mice. For IT experiments, pathogen-free 10–12-week-old male and female transgenic FVB/N mice expressing hFcyRI [14] and their nontransgenic littermates were used and maintained in biosafety level 3 facilities. For Cl2MBP-liposome experiments, pathogen-free 10-week-old female FVB/N mice were used (Harlan Sprague–Dawley). In all experiments, sex- and age-matched control mice were used. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

IT and Cl2MBP liposomes. The CD64 MAb H22 [15] was conjugated to deglycosylated ricin-A (Sigma) by use of heterobifunctional, cleavable N-succinimidyl 3-(2-pyridyldithio) propionate (Pierce), according to the manufacturers’ instructions. This antibody does not stain cross-reactively on cells from nontransgenic mice [16]. Treatment with IT results in complete clearance of CD64+ macrophages (activated macrophages) when it is injected intradermally in mice with chronic cutaneous inflammation [8]. The depletion of activated macrophages and leukemia cells by IT is accomplished by apoptosis [8, 9]. We found that intranasal (inl) administration of IT resulted in 41% macrophage depletion in lung homogenates of mice 7 h later, as assessed by fluorescence-associated cell sorting (FACS) analysis. Intratracheal administration of liposome-encapsulated Cl2MBP nonselectively depletes alveolar macrophages [10]. Cl2MBP was a gift from Roche Diagnostics. The preparation of liposomes containing Cl2MBP was done as described elsewhere [13]. We and others [2, 17, 18] have demonstrated that inl administration of liposome-encapsulated Cl2MBP resulted in >70% alveolar macrophage depletion in the bronchoalveolar lavage fluid of mice after 2 days. This depletion is induced by apoptosis [2, 11, 12].

Efficacy of treatment with IT and CD64 staining. To verify the efficacy of IT, FVB/N mice (n = 10) received 0.8 × 10⁶ U of recombinant murine interferon (IFN)–γ (CLB) inl twice, at 24 and 0 h relative to IT treatment, to up-regulate FcyRI expression on macrophages. At time 0, 25 μL of IT (2 × 10⁴ mol, referring to ricin-A moiety, in saline; n = 5) or saline (control mice; n = 5) was administered inl. After 7 h, mice were killed. Frozen lung sections (6 μm) were exposed to fluorescein isothiocyanate (FITC)–labeled mouse anti–human CD64 10.1 (Instruchemie), rabbit anti–FITC antibody (Dako), and goat anti-rabbit horseradish peroxidase antibody (Dako) and were developed by use of 1% H₂O₂ and amin-ethylcarbazole. Sections were counterstained with hematoxylin. The number of hCD64+ cells was counted in 3 mm² of sample (5 fields at ×40 magnification). Additionally, FACS analysis was performed on lung homogenates.

Detection of apoptosis. Tissue sections of left lungs were deparaffinized and boiled twice for 5 min each in citrate buffer (pH 6.0). Nonspecific binding and endogenous peroxidase activity were blocked as described above, followed by incubation with rabbit anti–human active caspase 3 polyclonal antibody (Cell Signaling) and biotinylated swine anti–rabbit antibody (Dako). Slides were incubated in streptavidin-ABC solution (Dako) and processed as described above. Additionally, the apoptosis of macrophages in lung homogenates was analyzed by FACS analysis.

Experimental infection and in vivo depletion of activated macrophages. TB was induced as described elsewhere [2, 19, 20]. Briefly, mice were anesthetized by inhalation with isofluorane (Abbott Laboratories) and infected inl with live M. tuberculosis H37Rv bacilli in 50 μL of saline. FVB/N mice appeared to be less susceptible to pulmonary M. tuberculosis infection than the frequently used C57BL/6 mice (authors’ unpublished observations). Therefore, mice were infected inl with 7 × 10⁵ cfu (IT experiments) or 25 × 10⁵ cfu (Cl2MBP-liposome experiments), to effectively mimic the infectious dose used in our previous study of the role of alveolar macrophages in host defense against TB in the lung [2]. To deplete activated macrophages, IT (25 μL) or saline (control) was administered inl twice weekly for 5 weeks, starting 2 days after M. tuberculosis challenge. As a control for hFcyRI mice treated with IT (activated macrophage–mice), transgenic hFcyRI mice were treated with saline, and their nontransgenic littermates were treated with either saline or IT (activated macrophage–mice). For nonselective depletion of macrophages, Cl2MBP liposomes (100 μL) were administered inl 4, 11, 21, and 29 days after M. tuberculosis challenge (macrophage–mice).
mice). Control mice received 100 μL of PBS and liposomes inl (macrophage− liposome-treated mice) or saline (macrophage− saline-treated mice). Mice (8/group) were killed 5 weeks after infection. Lungs and spleens were homogenized, and 10-fold serial dilutions were plated on Middlebrook 7H11 agar plates for the assessment of mycobacterial outgrowth. For cytokine measurements, lung homogenates were diluted in lysis buffer (150 mmol/L NaCl, 15 mmol/L Tris, 1 mmol/L MgCl2·H2O, 1 mmol/L CaCl2, 1% Triton, and 100 μg/mL pepstatin A, leupeptin, and aprotinin), and supernatants were sterilized by use of a 0.22-μm filter (Corning).

**FACS analysis.** Pulmonary cells were obtained by use of an automated disaggregation device (Medimachine System) and re-suspended in RPMI 1640 medium (BioWhittaker), 10% fetal calf serum, and 1% antibiotic-antimycotic (GibcoBRL), as described elsewhere [2]. Erythrocytes were lysed, and cells were brought to a concentration of 4 × 106 cells/mL. To determine the efficacy of IT, lung cells from 4 mice/group were immunostained with FITC-labeled mouse anti-human CD64 10.1 (Instruchemie) and CD11b-phycocerythrin (PE; Pharmingen) after gating on macrophages. Additionally, CD64-positive cells were stained with CD86-PE (Pharmingen), to identify dendritic cells (DCs). For the analysis of apoptotic macrophages, lung cells were gated for macrophages by forward and side scatter and stained with Annexin-FITC (Pharmingen) and 7-amino-actinomycin D (Phar-mingen). For each analysis of infected mice, lung cells from 2 animals/group (n = 8/group) were pooled. Immunostaining for cell-surface molecules was performed by use of antibodies against CD3 (anti-mCD3-PE), CD4 (anti-mCD4-CyChrome), CD8 (anti-mCD8-FITC, anti-mCD8–peridinin-chlorophyll-protein complex), and CD69 (anti-mCD69-FITC). Cells were fixed with 2% paraformaldehyde and analyzed by gating the CD3+ population.

**Enumeration of granulomas.** The right middle lung lobes were removed 5 weeks after inoculation with M. tuberculosis and fixed in 4% paraformaldehyde in PBS for 24 h. After they were embedded in paraffin, 4-μm-thick sections were stained with hematoxylin-eosin for histological analysis.

**Splenocyte and lymph-node cell stimulation.** Single-cell suspensions were obtained by crushing spleens or tracheobronchial lymph nodes through a 40-μm cell strainer (Merck). Erythrocytes were lysed, and cells were suspended in medium, seeded in 96-well culture plates at a cell density of 1 × 10⁶ cells in triplicate, and stimulated with tuberculin purified protein derivative (PPD; Statens Seruminstitut). Supernatants were harvested after 48 h at 37°C in 5% CO₂, and cytokine levels were analyzed by ELISA.

**Cytokine measurements.** IFN-γ, interleukin (IL)−4, tumor necrosis factor (TNF)−α, and IL-12p40 were measured by specific ELISAs by use of matched antibody pairs, according to the manufacturer’s instructions (R&D Systems).

**Statistical analysis.** All values are expressed as mean ± SE. Statistical analysis was conducted by use of the Kruskal-Wallis test to evaluate variance among >2 groups. If significant variance was found, a Mann-Whitney U test was used to determine significant differences between individual groups. P < .05 was considered to be statistically significant.

**RESULTS**

**Depletion of hFcγRII** macrophages. To confirm the capability of IT to deplete activated pulmonary macrophages in vivo, we treated mice inl with IFN-γ to up-regulate FcγRI on macrophages and administered either IT or saline. A single inl administration of IT resulted in >34% depletion of hCD64+ cells in lungs after 7 h, compared with IFN-γ and saline (figure 1A; P < .05). The 7-h time point was chosen on the basis of the results of a previous study in which U937 cells were stimulated with IFN-γ and incubated for 6 h with different concentrations of IT, to analyze the efficiency of this compound [8]. FACS analysis of lung homogenates revealed that the percentage of FcγRI-positive macrophages was 32% lower in mice treated with IFN-γ and IT, compared with that in mice treated with IFN-γ and saline. The administration of IT had no direct effect on the percentage of FcγRI-positive DCs, as was demonstrated by Thepen et al. [8] (figure 1B). The depletion of activated macrophages was accomplished by the induction of apoptosis, as demonstrated by the detection of active caspase 3 in lung tissue (figure 1C). This is in accordance with previous findings on the capability of IT to deplete activated macrophages by inducing apoptosis [8].

**FcγRI expression during TB.** To study the expression of FcγRI in lungs during M. tuberculosis infection, we stained lung tissue sections from hFcγRI mice. This revealed a 3-fold increase in the amount of FcγRI expression in transgenic mice infected for 5 weeks with M. tuberculosis, compared with the expression in uninfected transgenic mice (109.4 ± 5.9 vs. 36.0 ± 13.8 CD64+ cells/3 mm², respectively; P < .05).

**Bacterial burden.** To investigate the role of activated macrophages in the outcome of TB, CL,MBP and liposomes or IT were administered after the induction of TB, to deplete macrophage in a nonselective manner (macrophage− mice) or only activated macrophages (activated macrophage+ mice). Significant differences in the tissue content of M. tuberculosis bacilli were found between macrophage− saline-treated or macrophage+ liposome-treated and CL,MBP and liposome−treated (macrophage) mice 5 weeks after infection. Lungs of macrophage− mice contained 6-fold fewer viable mycobacteria than did those of macrophage− saline-treated mice (P < .05) and 3.4-fold fewer viable mycobacteria than did those of macrophage− liposome-treated mice (P < .05). Spleens of macrophage− mice contained 40% fewer disseminated bacilli than did those of macrophage+ liposome-treated mice (P < .05). In contrast, when activated macrophages were selectively depleted,
Figure 1. Effect of a single immunotoxin (IT) treatment on pulmonary activated macrophages. hFcγRI transgenic mice were in treated with interferon (IFN)-γ to activate macrophages, as indicated by the up-regulation of FcγRI (CD64), or with saline as a control. Additionally, IFN-γ–treated mice were in treated with IT to deplete activated pulmonary macrophages or with saline as a control. A, CD64+ cells identified 7 h later, by use of immunohistochemistry, in frozen lung-tissue sections. Data are mean no. of cells/3 mm² ± SE of 5 mice/group. B, CD11b+CD64+ and CD86+CD64+ cells identified by use of fluorescence-associated cell sorting analysis 7 h after IT treatment in lung homogenates. Data are mean % ± SE of 4 mice/group. Comparisons were done with Mann-Whitney U tests. †P<.05 vs. saline-treated mice; *P<.05 vs. mice treated with IFN-γ and saline. C, Apoptotic macrophages visualized by immunostaining lung tissue from IFN-γ–treated mice that received either saline or IT for active caspase 3 (original magnification, ×80).

numbers of mycobacteria in lungs of IT-treated hFcγRI activated macrophage− mice were 7-fold higher than those of IT-treated wild-type (wt) activated macrophage+ mice (figure 2; P<.05). Additionally, significantly more mycobacteria were recovered from lungs of IT-treated hFcγRI activated macrophage− mice than from saline-treated hFcγRI activated macrophage+ mice (P<.05). Furthermore, activated macrophage− mice had 2 and 3 times more mycobacteria in their spleens than did IT-treated wt activated macrophage+ and saline-treated hFcγRI activated macrophage+ mice, respectively (P<.05). The enhanced outgrowth of M. tuberculosis in activated macrophage− mice was confirmed in a second independent experiment (data not shown).

Cell subsets. Both macrophages and lymphocytes are crucial for the resolution of pulmonary M. tuberculosis infection [21]. Therefore, the number and phenotype of cells in lungs of 8 mice/group were assessed. A small but nonsignificant reduction in cell numbers was found in both macrophage− and activated macrophage− mice, compared with that in control mice. As expected, the percentage of macrophages in the lungs of CL2MBP and liposome–treated mice and IT-treated hFcγRI transgenic mice was lower, compared with that in control mice (table 1, only significant for the nonselective macrophage depletion). No significant differences were found in polymorphonuclear cell (PMN) and lymphocyte percentages between groups. Moreover, similar percentages of CD4+ and CD8+ T cells were present in lungs of macrophage− and macrophage+ saline- and liposome–treated mice and activated macrophage− and activated macrophage+ mice (table 2). CD4+ lymphocytes of macrophage− mice and CD4+ and CD8+ lymphocytes of activated macrophage− mice appeared to be more activated (CD69+) than lymphocytes of activated macrophage+ mice.

Formation of granulomas. We examined the development of granulomas in lungs of infected mice, because activated macrophages contribute to the formation of granulomas, and their presence is crucial for the containment and elimination of mycobacteria. Importantly, numbers of granulomas detected in lungs of activated macrophage− mice were significantly lower than those in activated macrophage+ mice (figure 3).

Cytokines in lungs. To assess the effect of the depletion of activated macrophage on cytokine profiles in the local inflammatory environment, we measured, by ELISA, IFN-γ as the typical type 1 cytokine and IL-4 as the typical type 2 cytokine in lungs. Levels of these cytokines were not significantly affected by the depletion of either nonselective or activated macrophages (data not shown). Interestingly, levels of TNF-α were significantly lower in activated macrophage− mice, compared with the other 3 groups (figure 4). Levels of IL-12 showed a similar trend, although this difference was not significantly different (data not shown). Levels of both TNF-α and IL-12 were not significantly different by the depletion of nonselective macrophages (data not shown).
Figure 2. Mycobacterial growth in lungs and spleens of mice nonselectively depleted of macrophages (A) and of mice depleted from activated macrophages (B) 5 weeks after infection with Mycobacterium tuberculosis H37Rv. Data are mean ± SE of 8 mice/group. Comparisons were done with Mann-Whitney U tests. Nonselective depletion of macrophages: *P < .05 vs. saline-treated macrophage+ mice (white bars); †P < .05 vs. liposome-treated macrophage+ mice (striped bars); ‡P < .05 vs. saline-treated macrophage+ mice. Depletion of activated macrophages: *P < .05 vs. immunotoxin (IT)–treated activated macrophage+ wild-type (wt) mice; †P < .05 vs. saline-treated activated macrophage+ hFcγRI mice; ‡P < .05 vs. saline-treated activated macrophage+ wild-type mice. Black bars, liposome-encapsulated dichloromethylene bisphosphonate–treated macrophage+ mice.

**Ex vivo–stimulated splenocytes and lymph-node cells.** To determine whether the effect of the depletion of macrophages extended to lymphoid tissue, the release of cytokines by T cells in infected mice was examined. Because IFN-γ is crucial to the protective response to M. tuberculosis infection [21], we stimulated splenocytes and draining lymph-node cells from infected animals with PPD and measured levels of this cytokine. Cells from tracheobronchial draining lymph nodes of macrophage+ mice secreted >2-fold more IFN-γ than did those of saline- or liposome-treated macrophage+ mice (figure 5; P < .05). A similar trend was found when splenocytes were stimulated with PPD. IL-4 was undetectable in supernatants of PPD-stimulated cells. When stimulated with αCD3/28, splenocytes of macrophage+ mice secreted 2 times less IL-4 than did those of liposome-treated macrophage+ mice (figure 5; P < .05). Although activated macrophage+ mice were less able to control M. tuberculosis infection than were control and macrophage+ mice, they were still able to produce IFN-γ in an Ag-specific recall

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells ×10⁶/mL</th>
<th>Macrophages, %</th>
<th>PMNs, %</th>
<th>Lymphocytes, %</th>
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<tr>
<td>Nonselective depletion of macrophages</td>
<td></td>
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<tr>
<td>Saline-treated macrophage+</td>
<td>2825 ± 323</td>
<td>24.4 ± 2.8</td>
<td>52.0 ± 1.6</td>
<td>22.1 ± 1.4</td>
</tr>
<tr>
<td>Liposome-treated macrophage</td>
<td>2750 ± 290</td>
<td>21.1 ± 2.9</td>
<td>55.9 ± 1.2</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>CI₂MBP liposome–treated macrophage+</td>
<td>2613 ± 162</td>
<td>12.6 ± 2.2α</td>
<td>54.0 ± 2.2</td>
<td>33.4 ± 4.2</td>
</tr>
<tr>
<td>Depletion of activated macrophages</td>
<td></td>
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<tr>
<td>Saline-treated activated macrophage+ WT</td>
<td>1886 ± 162</td>
<td>26.2 ± 2.3</td>
<td>45.1 ± 2.5</td>
<td>28.7 ± 1.8</td>
</tr>
<tr>
<td>Saline-treated activated macrophage+ hFcγRI</td>
<td>1295 ± 168</td>
<td>22.3 ± 2.2</td>
<td>49.7 ± 0.9</td>
<td>28.1 ± 2.6</td>
</tr>
<tr>
<td>IT-treated activated macrophage+ WT</td>
<td>1396 ± 221</td>
<td>24.3 ± 2.6</td>
<td>48.6 ± 3.0</td>
<td>27.0 ± 2.1</td>
</tr>
<tr>
<td>IT-treated activated macrophage+ hFcγRI</td>
<td>1065 ± 202b</td>
<td>19.4 ± 1.1</td>
<td>47.9 ± 3.9</td>
<td>31.7 ± 4.1</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. ± SE of leukocytes in lungs of mice infected with Mycobacterium tuberculosis 5 weeks after infection. Total nos. of cells from 2 mice were pooled (collected from 8 mice/group/time point) and stained with hematoxylin-eosin. Comparisons were done with Mann-Whitney U tests. CI₂MBP, dichloromethylene bisphosphonate; IT, immunotoxin; PMNs, polymorphonuclear cells; wt, wild type.

α P < .05 vs. saline-treated macrophage+ mice.

β P < .05 vs. saline-treated activated macrophage+ WT mice.
response (figure 6). Tracheobronchial lymph-node cells and splenocytes of activated macrophage+ mice produced 53% and 88%, respectively, more IFN-γ than IT-treated wt activated macrophage+ mice. The production of IL-4 by lymph-node cells in response to αCD3/28 was lower in activated macrophage+ mice than in activated macrophage+ IT-treated wt mice. Hence, overall, both macrophage− and activated macrophage+ mice displayed a polarized type-1 (IFN-γ) cytokine response on stimulation of either bronchotracheal draining lymph-node cells or splenocytes.

**DISCUSSION**

An effective host defense against pulmonary mycobacterial infection relies on fast clearance of microorganisms from the lungs. Macrophages have been implicated as the predominant inducers of protection by phagocytosing and killing mycobacteria and by initiating the inflammatory response. However, macrophages are also the primary host cells for mycobacteria. The present study clearly demonstrates that the dual role of macrophages in vivo is related to the activation state of these cells. Although a non-selective depletion of macrophages after infection with *M. tuberculosis* improved the clinical outcome of disease, a depletion of activated macrophages led to impaired resistance, as reflected by enhanced mycobacterial outgrowth. These results suggest that macrophages facilitate the growth of mycobacteria until they are activated. Apparently, resting macrophages provide a sanctuary for intracellular replication and hiding, whereas activated macrophages inhibit mycobacterial growth.

The protective role of macrophages during TB is supported by observations of an inverse correlation between virulence and the early events of the activation of macrophages. The major component of mycobacterial cell walls, lipoarabinomannan (LAM), from a virulent *M. tuberculosis* strain did not stimulate the early events in the activation of macrophages, in which LAM from an avirulent strain increased the expression of genes and cytokines involved in early activation of macrophages [22]. By avoiding the activation of macrophages, LAM from virulent *M. tuberculosis* may act as a virulence factor that reduces growth inhibition by the host. In addition, the deactivation of macrophages has been shown to contribute to the pathogenesis of mycobacterial infection [23]. Activated macrophages can inhibit mycobacterial growth by displaying several direct antimicrobial mechanisms, such as the delivery of lysosomal enzymes to phagosomes, the production of toxic effector molecules, and the deprivation of intracellular iron availability. Furthermore, activated macrophages are important for the production of cytokines, macrophage-lymphocyte contacts, the
formation of granuloma, and the attraction of PMNs. Indeed, the production of TNF-α and numbers of granulomas, both of which play a central role in the containment of disease, were lower in activated macrophage− mice than in activated macrophage+ mice. Additionally, despite the higher mycobacterial load, a trend was seen of reduced absolute numbers of PMNs and lymphocytes in the lungs of mice depleted of activated macrophages, compared with that in lungs of control mice. Moreover, activated macrophages are involved in a tissue-damaging immune response that limits M. tuberculosis growth by destroying nonactivated macrophages and by forming nonliquefied caseous foci [24, 25]. In these solid foci, bacilli do not replicate significantly, because of anoxic conditions, the presence of inhibitory fatty acids, and reduced pH [26, 27]. The opposite effects of depletion of nonselective and activated macrophages indicate that activated macrophages have a surplus value above the provision of a sanctuary for mycobacteria.

In agreement with the results of our previous study [2], we have found that nonselective induction of the apoptosis of macrophages during TB was beneficial to the host. Because we induced the apoptosis of macrophage after, instead of before, the infection, as in our previous study, a better reflection of the in vivo situation was achieved. In contrast, the apoptosis of activated macrophages during M. tuberculosis infection was detrimental to the host and beneficial to the pathogen. This raises questions regarding the exact role of the apoptosis of macrophages during mycobacterial infection. The tremendous apoptosis of macrophages found in patients with TB [28–30] could work in 2 different ways. On the one hand, it could be a host defense mechanism to destroy and restrict the growth of intracellular mycobacteria [31] by depriving mycobacteria of host cells [32] and by constraining mycobacteria to apoptotic bodies [31]. Indeed, the apoptosis of human monocytes has been shown to limit the growth of M. avium [32], M. bovis bacillus Calmette-Guérin [33], and M. tuberculosis [34] in vitro and to lead to a reduced outgrowth of M. tuberculosis in vivo [2] (present study). On the other hand, the apoptosis of mac-

**Figure 4.** Levels of tumor necrosis factor (TNF)−α in lungs of mice depleted of activated macrophages 5 weeks after infection with Mycobacterium tuberculosis H37Rv. Data are mean ± SE of 8 mice/group. Comparisons were done with Mann-Whitney U tests. ∗P<.05 vs. the other 3 groups. White bar, saline-treated activated macrophage+ wild-type (wt) mice; striped bar, saline-treated activated macrophage− hFcγR mice; dotted bar, immunotoxin (IT)−treated activated macrophage+ wt mice; black bar, IT-treated activated macrophage− hFcγR mice.

**Figure 5.** Levels of interferon (IFN)−γ and interleukin (IL)−4 in supernatants of splenocytes and tracheobronchial lymph-node cells from macrophage+ saline-treated (white bars) and liposome-treated (striped bars) mice and from macrophage− mice (black bars) in response to purified protein derivative (top) and αCD3/28 antibodies (bottom). Splenocytes were harvested 5 weeks later, inoculated with Mycobacterium tuberculosis, and stimulated for 48 h. Data are mean ± SE of 8 mice/group. Comparisons were done with Mann-Whitney U tests. ∗P<.05 vs. saline-treated macrophage+ mice; †P<.05 vs. liposome-treated macrophage+ mice.
Figure 6. Levels of interferon (IFN)–γ and interleukin (IL)–4 in supernatants of splenocytes and tracheobronchial lymph-node cells from activated macrophage+/saline-treated wild-type (wt; white bars) and hFcγRI (striped bars) mice, immunotoxin (IT)-treated activated macrophage+/wt mice (hatched bars), and IT-treated macrophage+/H11002 hFcγRI mice (black bars) in response to purified protein derivative (top) and αCD3/28 antibodies (bottom). Splenocytes were harvested 5 weeks later, inoculated with Mycobacterium tuberculosis, and stimulated for 48 h. Data are mean ± SE of 8 mice/group. Comparisons were done with Mann-Whitney U tests. * vs. IT–treated activated macrophage+/wild-type (wt) mice; ‡ vs. saline-treated activated macrophage+/hFcγRI mice; † vs. saline-treated activated macrophage+/wt mice.

Rolphages could also be a weapon of M. tuberculosis to deprive the host of antimicrobial phagocytes and to impair cell-mediated immunity as far as it concerns activated macrophages. In this way, the apoptosis of activated macrophages seems to be an improbable strategy for host defense, and it could be a determinant of pathogen-encoded virulence. Interestingly, activated macrophages showed a higher level of apoptosis after infection with Salmonella typhimurium, a bacterium that also invades macrophages [35]. This could mean that the apoptosis of activated macrophages is indeed associated with pathogen-encoded virulence determinants and that the capability to induce apoptosis in activated macrophages may be important for the survival of the bacterium and its escape from the host immune response. One must, however, keep in mind that the mechanism of ricin-induced apoptosis might be different from the mechanism of naturally occurring apoptosis during M. tuberculosis infection, which might influence the survival of mycobacteria.

The other major effector cells involved in cell-mediated immunity to TB are lymphocytes. Macrophages have been traditionally recognized to be important antigen-presenting cells for CD4+ lymphocytes. However, the depletion of neither non-selective nor activated macrophages lowered the percentages of activated T cells. This suggests that macrophages in the lungs have poor antigen-presenting capabilities, which has been reported elsewhere [36–39]. Apparently, pulmonary macrophages are more important for phagocytosis and the clearance of mycobacteria without signaling for an amplification of the host inflammatory response. Alveolar macrophages have even been suggested to inhibit the local augmentation of the inflammatory response [40] and to suppress T cell proliferation to mycobacterial antigens [41–43]. In this way, macrophages can clear antigens without activating a host immune response, which can result in damaged alveolar capillary membranes [44].

During M. tuberculosis infection, CD8+ T cells have as their main function the lysis of infected cells and the production of cytokines [21]. Interestingly, we found relatively more activated CD8+ T cells in activated macrophage− mice than in activated macrophage+ mice. Because CD8+ T cells can be activated by secreted cytokines of activated CD4+ T cells, one could imagine that the relative increase in activated CD4+ T cells in activated macrophage− mice will lead to an increase in CD8+ T cell activity. Indeed, during HIV infection, the reduced numbers of CD4+ T cells may also result in an incomplete activation of CD8+ T cells [45]. CD8+ T cells can be activated by DCs. A further explanation could therefore be found in an enhancement of the antigen-presenting capabilities of DCs—that is, the antigen-presenting function of pulmonary DCs can be down-regulated by alveolar macrophages [46, 47]. More data are needed to analyze the kinetics of the cellular response more thoroughly.

In accordance with the results of our previous study [2], we found that the immune response of stimulated splenocytes and lymph-node cells of activated macrophage− mice was predominated by a type 1 response, compared with activated macrophage+ mice. It could be that alveolar macrophages exert Th2-associated functions and induce the differentiation of naïve T cells into Th2-type cells [48, 49]. Although the differences in
pulmonary leukocyte subsets and cytokine production by lymphoid cells are interesting, the relative contribution of this in mycobacterial resistance is unknown—that is, both macrophage-activated and activated macrophage mice displayed increased numbers of activated lymphocytes and increased type-I responses in lymphoid tissue, whereas macrophage-mice had a decreased mycobacterial load and activated macrophage-mice had increased mycobacterial outgrowth in their lungs. This observation emphasizes the important role that innate immunity plays in the host defense against pulmonary mycobacterial infection.

In summary, we have shown that, although resting macrophages provide a sanctuary for replicating mycobacteria, activated macrophages are important for the control of pulmonary TB infection. In addition, we have found that the antimicrobial effect of macrophages going into apoptosis depends on the activation state of the cell. The presence of the apoptosis of macrophages could be part of a host defense strategy against mycobacteria, as long as these cells are not activated. The present data demonstrate that innate immunity plays a key role in the defense against M. tuberculosis infection.

Acknowledgments

We thank J. Daalhuijzen, L. Bevaart, and A. J. van Vuuren, for expert technical assistance and advice; and J. Langermans, for giving us the opportunity to process the lung-tissue samples.

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