Pulmonary immune response during (myco)bacterial infection
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CHAPTER 3

Dual Role of Macrophages During Pulmonary Tuberculosis in Mice

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Pulmonary macrophages (Mφs) provide the preferred hiding and replication site for Mycobacterium tuberculosis bacilli. Paradoxally, Mφs display several very potent antimicrobial effector functions to eliminate mycobacteria. This raises questions as to the exact role of Mφs and Mφ apoptosis during mycobacterial infection. We therefore depleted lungs of activated Mφs by treating M. tuberculosis-infected transgenic mice expressing human FcγRI (CD64), which is upregulated upon activation, intranasally with an apoptosis-inducing antibody directed against FcγRI conjugated to Ricin-A (activated Mφ- mice). For comparison with unselective Mφ depletion, mice were intranasally inoculated with liposome-encapsulated dichloromethylene bisphosphonate (Mφ- mice). Whereas, a non-selective depletion of Mφs after infection with M. tuberculosis improved the clinical outcome of disease, a depletion of activated Mφs led to an impaired resistance as reflected by an enhanced mycobacterial outgrowth. Both Mφ- and activated Mφ- mice had a polarized production of the type 1 cytokine IFNγ by splenocytes and lymph node cells stimulated ex vivo and were fully capable of attraction and activation of T cells into the lung. These data demonstrate that the dual role of macrophages in vivo is associated with the activation state of Mφs and that the extensive Mφ apoptosis found in tuberculosis patients could be part of a host defense strategy, as long as these cells are not activated.
INTRODUCTION

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 infections is the intracellular pathogen *Mycobacterium tuberculosis*, which causes more deaths annually than any other single infectious agent (1). Since *M. tuberculosis* replicates predominantly in Mφs, its pathogenicity is critically dependent on the outcome of the interaction between the Mφ and bacterium. There is evidence that Mφs can destroy *M. tuberculosis* by different mechanisms. They can deliver lysosomal enzymes to phagosomes, produce toxic effector molecules like reactive nitrogen intermediates, limit the iron availability for mycobacteria and undergo apoptosis. Paradoxically, Mφs are also the main host cells for *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 Mφs may facilitate the growth of mycobacteria in the lung, we recently found that ~70% depletion of alveolar Mφs before infection with *M. tuberculosis* completely protected mice against lethality and led to an attenuated mycobacterial outgrowth (2). Together this led us to hypothesize that Mφs play a dual role during *M. tuberculosis* infection. It can be anticipated that the activation state of Mφs is important in determining whether these cells facilitate or inhibit the growth of mycobacteria. Indeed, Mφs activated *in vitro* by cytokine treatment have been demonstrated to inhibit the growth of mycobacteria (3), although others reported that this might be an artefact (4). To study the *in vivo* correlation of Mφ activation with antimicrobial functions, Mφ activating and inactivating cytokines are often used (5).

Since the exact timing, combination and sequence of signals needed for sufficient Mφ activation are not identified, we used a different approach to determine the influence of the activation state of Mφs on their role in tuberculosis *in vivo*. We depleted mice of activated pulmonary Mφs by inducing apoptosis in these cells. For this purpose we used transgenic mice expressing human FcγRI (CD64), which is upregulated upon activation (6, 7), and treated them with anti-human FcγRI mAb conjugated to Ricin-A after *M. tuberculosis* infection. In a previous study Thepen *et al.* demonstrated that this
immunotoxin (IT) targeted only activated inflammatory Mφs, leaving non-activated, low FcγRI-expressing cells unaffected (8). Additionally, it was demonstrated that this IT depletes activated Mφs and leukemia cells by inducing apoptosis (8, 9). For comparison, we as well determined the role of non-selective Mφ depletion after *M. tuberculosis* infection in mice, using the well-validated method of intrapulmonary delivery of liposome-encapsulated dichloromethylene bisphosphonate (Cl₂MBP).

Considering this we studied the *in vivo* role of activated Mφs in the host defense against *M. tuberculosis* and determined whether the (mycobactericidal) effects of non-selective depletion of Mφs by apoptosis are distinct from those of apoptosis induction in activated Mφs.

**MATERIAL & METHODS**

**Mice**

For IT experiments pathogen-free 10-12 week old male and female transgenic FVB/N mice expressing human FcγRI mice (10) and non-transgenic littermates were used and maintained in biosafety level 3 facilities. For Cl₂MBP-liposome experiments pathogen-free 10-week-old female FVB/N mice were obtained from Harlan Sprague Dawley Inc. (Horst, the Netherlands). In all experiments, sex and age matched controls were used. The Animal Care and Use Committee of the University of Amsterdam, the Netherlands, approved all experiments.

**Immunotoxin (IT) and Cl₂MBP-liposomes**

The CD64 monoclonal antibody (Mab) H22 (11) was conjugated to deglycosylated Ricin-A (Sigma, St. Louis, MO), using the heterobifunctional, cleavable N-succinimidyl 3-(2-pyrydylthio) propionate (SPDP) (Pierce, Rockford, IL) according to the manufacturers’ instructions. Conjugates were purified using size exclusion chromatography and purity was checked on SDS-PAGE gradient gel. This IT was shown to result in a complete clearance of high CD64 expressing Mφs (activated Mφs) when injected intradermally in mice with a chronic cutaneous inflammation (8). Depletion of activated Mφs and leukemia cells by this IT has been shown to be accomplished by the induction of apoptosis (8, 9). Intratracheal administration of liposome-encapsulated Cl₂MBP non-
selectively depletes alveolar M\(\phi\)s (12). Cl\(_2\)MBP was a gift from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing Cl\(_2\)MBP was done as described previously (13). We and others (2, 14, 15) have demonstrated that intranasal administration of liposome-encapsulated CL\(_2\)MBP resulted in >70% alveolar M\(\phi\) depletion in BALF of mice after 2 days. This depletion has been shown to be induced by apoptosis (2, 16, 17).

Efficiency of IT treatment/CD64 staining

To verify the cytotoxic efficacy of the IT, FVB/N mice (n=10) received 0.8×10^6 U recombinant murine IFN\(\gamma\) (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands) intranasally (i.n.) twice at -24 h and 0 h relative to IT treatment to upregulate FcyRI expression on M\(\phi\)s. At t=0, 25 \(\mu\)l IT (2 x 10\(^7\) M referring to the Ricin-A moiety, in saline; n=5) or saline (controls; n=5) were i.n. administered. After 7 h mice were sacrificed, lungs were removed, snap frozen in liquid nitrogen, and stored at -70°C prior to use. For histologic examination, lungs were cut into 6-\(\mu\)m sections and fixed for 10 min with ice-cold acetone and air-dried. Endogenous peroxidase activity was quenched by a solution of 10% NaN\(_3\)/0.03% H\(_2\)O\(_2\) (Merck, Darmstadt, Germany) in PBS. The sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) for 10 min and then exposed for 45 h to FITC-labeled mouse anti-human CD64 10.1 (Instruchemie, Delfzijl, The Netherlands; 1:40) in PBS with 10% normal goat serum. Slides were incubated with a rabbit anti-FITC antibody (Dako) followed by a further incubation with a goat anti-rabbit HRP antibody (Dako), rinsed again, and developed using 1% H\(_2\)O\(_2\) and AEC. The sections were slightly counterstained with hematoxylin. The amount of hCD64+ cells was counted in 3 mm\(^2\) (5 fields of x40).

Detection of apoptosis

Tissue sections of left lungs were deparaffinized and boiled 2 x 5 min in citrate buffer (pH 6.0). Non-specific binding and endogenous peroxidase activity were blocked as described, followed by an incubation with rabbit anti-human active caspase 3 polyclonal antibody (Cell Signaling, Beverly, MA) followed by a further incubation with a goat anti-rabbit HRP antibody (Dako), rinsed again, and developed using 1% H\(_2\)O\(_2\) and AEC. The sections were slightly counterstained with hematoxylin. The amount of hCD64+ cells was counted in 3 mm\(^2\) (5 fields of x40).
**Experimental infection and in vivo depletion of (activated) Mφs**

Tuberculosis was induced as described previously (2, 18). Briefly, a virulent laboratory strain of *M. tuberculosis* H37Rv was grown in liquid Dubos medium containing 0.01% 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 anaesthetized by inhalation with isoflurane (Abbott Laboratories Ltd., Kent, U.K.) and i.n. infected with $7 \times 10^5$ (IT experiments) or $25 \times 10^5$ (Cl₂MBP-liposome experiments) live bacilli in 50 µl saline, as determined by viable counts on 7H11 Middlebrook agar plates. To deplete activated Mφs, IT (25 µl) or saline (control) was given i.n. twice a week for five weeks, starting 2 days after mycobacterial challenge. As a control for IT-treated hFcγRI mice (activated Mφ- mice), transgenic hFcγRI mice were treated with saline and non-transgenic littermates were treated with either saline or IT (activated Mφ+ mice). For non-selective Mφ depletion, Cl₂MBP-liposomes (100 µl) was administered i.n. 4, 11, 21, 29 days after *M. tuberculosis* challenge (Mφ- mice). Control mice received i.n. 100µl PBS-liposomes (Mφ+ (liposomes) mice) or saline (Mφ+ (saline) mice). Groups of eight mice per time point were sacrificed 5 weeks post-infection, and lungs, and spleen were removed aseptically. Organs were homogenized with a tissue homogenizer (Biospec Products, Bartlesville, OK) in 5 volumes 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 colony forming units (CFUs) are provided as total in the lungs or as total per gram spleen. For cytokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150mM NaCl, 15mM Tris, 1mM MgCl₂,H₂O, 1mM CaCl₂ 1% Triton, 100µg/ml pepstatin A, leupeptin and aprotinin), and incubated for 30 min. on ice. Supernatants were sterilized using a 0.22 µm filter (Corning Incorporated, Corning, NY) and frozen at -20°C until assays were performed.

**FACS analysis**

Lung cells obtained from infected mice were analyzed by FACS (Becton Dickinson, Franklin Lakes, NJ). 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% fetal calf serum, 1%
antibiotic-antimycotic (GibcoBRL, Life Technologies, Rockville, MD)) as described previously (2). Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4), and the remaining cells were washed twice with RPMI. For each analysis lung cells from 2 mice from a group (n=8 per group) were pooled, yielding 4 separate measurements per group at each time point. Cells were brought to a concentration of 4x10⁶ cells/ml FACS buffer (PBS supplement with 0.5% BSA, 0.01% NaN₃ and 100 mM EDTA). Immunostaining for cell surface molecules was performed for 30 min at 4°C using Abs against CD3 (anti-mCD3-phycoerythrin), CD4 (anti-mCD4-CyChrome), CD8 (anti-mCD8-FITC, anti-mCD8-PerCP), CD69 (anti-mCD69-FITC). All Abs were used in concentrations recommended by the manufacturer (Pharmingen, San Diego, CA). To correct for aspecific staining an appropriate control antibody (rat IgG₂, Pharmingen) was used. Cells were fixed with 2% paraformaldehyde, and surface molecules were analyzed by gating the CD3⁺ population. The number of positive cells was obtained by setting a quadrant marker for nonspecific staining.

**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 with ice-cold isotonic NH₄Cl solution, and the remaining cells were washed twice. Cells were suspended in medium, seeded in 96-well round bottom culture plates at a cell density of 1x10⁶ cells in triplicate, and stimulated with tuberculin-purified protein derivative (PPD) (Statens Seruminstitut, Copenhagen, Denmark). Supernatants were harvested after a 48-h incubation at 37°C in 5% CO₂, and cytokine levels were analyzed by ELISA.

**Cytokine measurements**

IFNγ and IL-4 were measured in lung homogenates, spleen and lymph node cell supernatants by specific ELISA’s using matched Ab pairs according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

**Data statistical analysis**

All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests. Values of P < 0.05 were considered statistically significant.
RESULTS

Depletion of hFcyRI+ Mps

To confirm the capability of the IT to deplete activated pulmonary Mps in vivo we treated mice i.n. with IFNγ to upregulate FcyRI on Mps and administered either IT or saline. A single i.n. administration of IT resulted in >34% depletion of hCD64+ cells in lungs after 7h when compared to IFNγ- saline-treated animals (Fig. 1A, P<.05). Depletion of activated Mps was accomplished by the induction of apoptosis, as demonstrated by the detection of active caspase-3 in lung tissue (Fig. 1B). This is in line with previous findings on the capability of IT to deplete activated Mps by inducing apoptosis (8).

![Graph showing CD64+ cells](image)

**Figure 1** A. Effect of a single IT treatment on pulmonary activated Mps. hFcyRI transgenic mice were i.n. treated with IFNγ to activate Mps as indicated by an upregulation of FcyRI (CD64) or with saline as a control. Additionally, IFNγ-treated mice were i.n. treated with IT to deplete activated pulmonary Mps or with saline as a control. CD64+ cells were identified 7 h later in frozen lung tissue sections by immunohistochemistry. Data are mean number of cells/3mm² ± SEM of five mice per group. †, P<.05 vs saline-treated mice; *, P<.05 vs IFNγ- saline-treated mice. B. Apoptotic Mps were visualized by immunostaining lung tissue from IFNγ-treated mice that received IT for active caspase-3 (original magnification x80).

FcyRI expression during tuberculosis

To study the FcyRI expression in lungs during M. tuberculosis infection we stained lung tissue sections of hFcyRI mice. This revealed a 3-fold increase in the amount of FcyRI-expressing in transgenic mice infected for 5 wk with M. tuberculosis when compared to 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) Mps in the outcome of tuberculosis, CL2MBP-
liposomes or IT were given *after* induction of tuberculosis to respectively deplete Mφ in a non-selective manner (Mφ-) or to deplete activated Mφs (activated Mφ-). The ability of mice to limit the mycobacterial growth was studied 5 wks post-infection (p.i.). Significant differences in tissue content of *M. tuberculosis* bacilli were found between saline/liposome-treated (Mφ+) and CL2,MBP-liposome-treated animals (Mφ-). The lungs of Mφ- mice contained 6-fold less viable mycobacteria than those of Mφ+ (saline) animals (*P* <.05) and 3.4-fold less than of Mφ+ (liposomes) mice (Fig. 2, *P* <.05). The spleens of Mφ- mice contained 40% less disseminated bacilli than in Mφ+ (liposomes) mice (*P* <.05). In contrast, when activated Mφs were selectively depleted, the number of mycobacteria isolated from the lungs of IT-treated hFcyRI mice (activated Mφ-) was 7-fold higher compared to their IT-treated Wt mice (activated-Mφ+) (Fig. 2, *P* <.05). In addition, significantly more mycobacteria were recovered from lungs of IT-treated hFcyRI mice (activated Mφ-) than from saline-treated hFcyRI mice (activated Mφ+) (*P* <.05). Furthermore, IT-treated hFcyRI mice (activated Mφ-) had 2 and 3 times more mycobacteria in their spleens when compared to respectively IT-treated Wt mice (activated Mφ+) and saline-treated hFcyRI mice (activated Mφ+) (*P* <.05). The enhanced outgrowth of *M. tuberculosis* in IT-treated hFcyRI mice (activated Mφ-) was confirmed in a second independent experiment (data not shown).

**Figure 2** Mycobacterial growth in lungs and spleens of mice unselectively depleted from Mφs (A) and of mice depleted from activated Mφs (B) 5 wk after infection with H37Rv *M. tuberculosis*. Data are mean and SEM of eight mice per group. Unselective Mφ depletion: *+, *P* <.05 vs Mφ+ (saline) mice; †, *P* <.05 vs Mφ+ (liposomes) mice; ‡, *P* <.05 vs Mφ+ (saline) mice. Activated Mφ depletion: *+, *P* <.05 vs activated Mφ+ (IT-treated Wt) mice; †, *P* <.05 vs activated Mφ+ (saline-treated hFcyRI) mice; ‡, *P* <.05 vs activated Mφ+ (saline-treated Wt) mice.
Cell subsets

Both Mφs and lymphocytes are crucial for the resolution of pulmonary *M. tuberculosis* infection (19). Therefore, the cell number and phenotype of the cells in lungs of 8 mice per group were assessed. A small but non significant reduction in cell numbers in lungs was found in both Mφ- mice and activated Mφ- mice (IT-treated hFcγRI) as compared to their respective control mice. As expected the percentage of Mφ in the lungs of Cl2MBP-liposome-treated mice and IT-treated hFcγRI transgenic mice was lower when compared to their controls (Table I, only significant for the non-selective Mφ depletion). No significant differences were found in PMN and lymphocyte percentages between groups. Throughout the course of the infection, similar percentages CD4+ and CD8+ T cells were present in lungs of both Mφ- mice and Mφ+ (saline/liposomes) mice and activated Mφ- and activated Mφ+ mice (Table II). To investigate their activation state, T cells were stained for the activation marker CD69. CD4+ lymphocytes of Mφ- mice and CD4+ and CD8+ lymphocytes of activated Mφ- mice were demonstrated to be more activated than lymphocytes of (activated) Mφ+ mice.

Table I. Effect of (activated) Mφ depletion on cellular composition in lungs during tuberculosis

<table>
<thead>
<tr>
<th>non-selective Mφ depletion</th>
<th>Cells x10^6/ml</th>
<th>Mφ %</th>
<th>PMN %</th>
<th>Lymphocytes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mφ+ (saline)</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>Mφ+ (liposomes)</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>Mφ- (Cl2MBP-liposomes)</td>
<td>2613 ± 162</td>
<td>12.6 ± 2.2*</td>
<td>54.0 ± 2.2</td>
<td>33.4 ± 4.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>activated Mφ depletion</th>
<th>Cells x10^6/ml</th>
<th>Mφ %</th>
<th>PMN %</th>
<th>Lymphocytes %</th>
</tr>
</thead>
<tbody>
<tr>
<td>activated Mφ+ (saline-treated Wt mice)</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>activated Mφ+ (saline-treated hFcγRI mice)</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>activated Mφ+ (IT-treated Wt mice)</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>activated Mφ- (IT-treated hFcγRI mice)</td>
<td>1065 ± 202*</td>
<td>19.4 ± 1.1</td>
<td>47.9 ± 3.9</td>
<td>31.7 ± 4.1</td>
</tr>
</tbody>
</table>

*Leukocytes in lungs of mice infected with *M. tuberculosis* 5 weeks p.i.. Total cells from 2 mice were pooled (collected from eight mice per group per time point) and HE stained. *P < 0.05 vs Mφ+ (saline) mice; † P<.05 vs activated Mφ+ (saline-treated Wt) mice.

Cytokines in lungs

To assess the effect of (activated) Mφ depletion on cytokine profiles in the local inflammatory environment, we measured IFNγ as the typical type 1 cytokine and IL-4 as the typical type 2 cytokine in lungs by ELISA. Cytokine levels were not significantly affected by either non-selective Mφ or activated Mφ depletion (data not shown).
Table II. Effect of Mφ depletion on cell subsets in lungs during tuberculosis

<table>
<thead>
<tr>
<th>non-selective Mφ depletion</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>CD4⁺/CD69⁺</th>
<th>CD8⁺/CD69⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mφ⁺ (saline)</td>
<td>61.0 ± 2.6</td>
<td>30.3 ± 2.1</td>
<td>27.6 ± 1.4</td>
<td>22.0 ± 1.9</td>
</tr>
<tr>
<td>Mφ⁺ (liposomes)</td>
<td>61.2 ± 1.7</td>
<td>31.3 ± 2.0</td>
<td>32.4 ± 3.4</td>
<td>21.3 ± 1.1</td>
</tr>
<tr>
<td>Mφ⁻ (Cl₃MBP-liposomes)</td>
<td>60.1 ± 1.9</td>
<td>32.5 ± 1.8</td>
<td>36.3 ± 5.0*</td>
<td>23.7 ± 1.1</td>
</tr>
</tbody>
</table>

activated Mφ depletion

| activated Mφ⁺ (saline-treated Wt mice)                  | 68.5 ± 3.1 | 32.9 ± 3.4 | 23.0 ± 0.5 | 13.8 ± 2.5 |
| activated Mφ⁺ (saline-treated hFcγRI mice)             | 69.6 ± 1.0 | 31.3 ± 3.1 | 33.8 ± 2.3 | 16.3 ± 4.9 |
| activated Mφ⁺ (TT-treated Wt mice)                      | 73.3 ± 1.5 | 33.7 ± 4.0 | 27.9 ± 1.1 t | 8.9 ± 2.9 |
| activated Mφ⁻ (TT-treated hFcγRI mice)                 | 67.0 ± 4.6 | 38.0 ± 3.6 | 33.0 ± 3.3 * t | 22.3 ± 3.4 * t |

* Cell subsets in lungs of mice infected with *M. tuberculosis* 5 weeks post-infection. Total cells from 2 mice were pooled (collected from eight mice per group per time point) and immunostained. FACS analysis was performed as described in the methods section. Results are expressed as % CD4⁺, CD8⁺, and CD69⁺ within the CD3⁺ population. Non-selective Mφ depletion; * P<.05 vs Mφ⁺ (saline) mice. Activated Mφ depletion; * P<.05 vs activated Mφ⁺ (IT-treated Wt mice), t P<.05 vs activated Mφ⁺ (saline-treated Wt) mice.

*Ex vivo stimulated splenocytes and lymph node cells*

To determine if the effect of Mφ depletion extended to lymphoid tissue, T cell cytokine release in infected mice was examined. Since IFNγ is crucial to the protective response to *M. tuberculosis* infection (19), we stimulated splenocytes and draining lymph node cells from infected animals with a recall antigen PPD, and measured this cytokine. Cells from the tracheobronchial draining lymph nodes of Mφ⁻ mice secreted over two-fold more IFNγ than those of Mφ⁺ (saline/liposomes) mice (Fig. 3, P<.05). A similar tend was found when splenocytes were PPD-stimulated. IL-4 was undetectable in supernatants of

Figure 3 Levels of IFNγ and IL-4 in supernatants of splenocytes and tracheobronchial lymph node cells from Mφ⁺ (saline) mice, Mφ⁺ (liposomes) mice, and Mφ⁻ mice in response to PPD and aCD3/28 Abs. Splenocytes were harvested 5 wk after i.n. inoculation of *M. tuberculosis*, and stimulated for 48 h. Data are mean ± SEM of eight mice per group. *, P<.05 vs Mφ⁺ (saline) mice; t, P<.05 vs Mφ⁺ (liposomes) mice.
cells stimulated with PPD. When stimulated with αCD3/28 splenocytes of Mφ- mice secreted 2 times less IL-4 than Mφ+ (liposomes) mice (Fig. 3, P<.05). Although activated Mφ- mice were less able to control *M. tuberculosis* infection as their controls and Mφ+ mice they were still able to produce IFNγ in an Ag-specific recall response (Fig. 4). Tracheobronchial lymph node cells and splenocytes of activated Mφ- mice produced respectively 53% and 88% more IFNγ than activated Mφ+ (IT-treated Wt) mice. IL-4 production of lymph node cells in response to αCD3/28 was lower in activated Mφ+ mice when compared to activated Mφ+ (IT-treated Wt) mice. Hence, overall both Mφ- and activated Mφ- mice displayed a polarized type 1 (IFNγ) cytokine response upon stimulation of either bronchotracheal draining lymph node cells or splenocytes.

**DISCUSSION**

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

The protective role of Mφs during tuberculosis is supported by observations of an inverse correlation between virulence and the early events of Mφ activation. The major component of mycobacterial cell walls lipoarabinomannan (LAM) from a virulent *M. tuberculosis* strain failed to stimulate the early events in macrophage activation where LAM from an avirulent strain increased the expression of genes and cytokines which are involved in early Mφ activation (20). By avoiding Mφ activation, LAM from virulent *M. tuberculosis* may act as a virulence factor that reduces growth inhibition by the host. In addition, Mφ deactivation has been shown to contribute to the pathogenesis of mycobacterial infection (21). Activated Mφs can inhibit mycobacterial growth by displaying several direct antimicrobial mechanisms, like the delivery of lysosomal enzymes to phagosomes, the production of toxic effector molecules, and the deprivation of the intracellular iron availability. Furthermore, activated Mφs are important for the production of cytokines, Mφ-lymphocyte contacts, the formation of granulomas, and the attraction of PMNs. Indeed, despite the higher mycobacterial load a trend was seen of reduced absolute numbers of PMNs and lymphocytes in lungs of mice depleted from activated Mφs compared to lungs of their controls. Additionally, activated Mφs are involved in a tissue damaging immune response that limits the growth of *M. tuberculosis* by destroying non-activated Mφs and by forming nonliquefied caseous foci (22, 23). In these solid foci tubercle bacilli do not replicate significantly because of anoxic conditions, the presence of inhibitory fatty acids and a reduced pH (24, 25). The opposite effects of unselective and activated Mφ depletion indicate that activated Mφs have a surplus value above the provision of a sanctuary for mycobacteria.

In agreement with our previous study (2), we found that a non-selective induction of Mφ apoptosis during tuberculosis was beneficial for the host. Since we induced Mφ apoptosis after instead of before the infection as in our previous study, a better reflection of the *in vivo* situation is achieved. In contrast, the induction of apoptosis of
activated Mφs during *M. tuberculosis* infection was detrimental for the host and beneficial for the pathogen. This raises questions as to the exact role of Mφ apoptosis during mycobacterial infection. The tremendous Mφ apoptosis found in tuberculosis patients (26-28) could work in two totally different ways. On the one hand it could be a host defense mechanism to destroy and restrict the growth of intracellular mycobacteria (29) by depriving mycobacteria from host cells (30) and by constraining of mycobacteria in apoptotic bodies (29). Indeed, apoptosis of human monocytes has been shown to limit the growth of *M. avium* (30), *M. bovis* bacillus Calmette-Guérin (31) and *M. tuberculosis* (32) *in vitro* and led to a reduced outgrowth of *M. tuberculosis in vivo* (2) (this study). However, Mφ apoptosis could also be a weapon of *M. tuberculosis* to deprive the host of antimicrobial phagocytes and impair with cell-mediated immunity as long as it concerns activated Mφs. In this way activated Mφs apoptosis seems an improbable strategy for host defense and could be rather a pathogen-encoded virulence determinant. Interestingly, activated Mφs showed a higher level of apoptosis after infection with *Salmonella typhimurium*, a bacterium that also invades Mφs (33). This could mean that apoptosis of activated Mφ is indeed associated with pathogen-encoded virulence determinants and that the capability to induce apoptosis in activated Mφs may be important for the survival of the bacterium and escape of the host immune response.

Other major effector cells in cell-mediated immunity to tuberculosis are lymphocytes. Mφs have been 'classically' recognized as important antigen-presenting cells for CD4⁺ lymphocytes. However, neither non-selective Mφ nor activated Mφs depletion during tuberculosis lowered the percentages of activated T cells. (Activated) Mφ depletion even led to a slightly increased percentage of activated T cells as assessed by the activation marker CD69. This suggests that Mφs residing in the pulmonary compartment have poor antigen-presenting capabilities, which is underlined by several other reports (34-37). Apparently, pulmonary Mφs are more important for the phagocytosis and clearance of mycobacteria without signaling for an amplification of the host inflammatory response. Alveolar Mφs are even suggested to inhibit the local augmentation of the inflammatory response (38) and to suppress T cell proliferation to mycobacterial antigens (39-41). In this way Mφs can clear antigens without activating a host immune response that can result in damaged alveolar capillary membranes (42).

During *M. tuberculosis* infection CD8⁺ T cells have as main effector function the lysis of infected cells and the production of cytokines, in particular IFNγ (19).
Interestingly we found relatively more activated CD8⁺ T cells in activated Mφ- mice compared to activated Mφ+ mice. Since 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 Mφ- animals finally will lead to an increase in CD8⁺ T cell activity. Indeed, during HIV infections the reduced numbers of CD4⁺ T cells may also result in an incomplete activation of CD8⁺ T cells (43). In addition, CD8⁺ T cells can be activated by dendritic cells (DCs). A further explanation for the increase in activated CD8⁺ T cells could therefore be found in an enhancement of the antigen presenting capabilities of DCs in activated Mφ- mice, i.e. the antigen presenting function of pulmonary DCs can be downregulated by alveolar Mφs (44, 45).

In line with our previous study (2), we found that the immune response of splenocytes and lymph node cells of (activated) Mφ- mice in response to PPD and anti-CD3/28 was predominated by a type 1 response when compared to (activated) Mφ+ mice. It could be that alveolar Mφs exert Th2-associated effector functions and induce the differentiation of naïve T cells into Th2 type cells (46, 47). Although the differences in pulmonary leukocyte subsets and the polarized production of type 1 cytokines by splenocytes and lymph node cells of (activated) Mφ- mice compared to (activated) Mφ+ mice are interesting, the relative contribution of this in the mycobacterial resistance is unknown. That is both Mφ- and activated Mφ- mice displayed increased (activated) lymphocyte numbers and type 1 responses in lymphoid tissue while Mφ- mice had a decreased mycobacterial load and activated Mφ- mice had an increased mycobacterial outgrowth in their lungs when compared to their respective control mice.

In summary, we here demonstrate that whereas resting Mφs provide a save sanctuary for replicating mycobacteria, activated Mφs are important for the control of pulmonary tuberculosis infection. In addition, we found that the antimicrobial effect of Mφs going into apoptosis depends on the activation state of this cell. The presence of Mφ apoptosis could be part of a host defense strategy against mycobacteria, as long as these cells are not activated.

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