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Culture at High Density Improves the Ability of Human Macrophages to Control Mycobacterial Growth

Neio Boechat,* Francine Bouchonnet,* Marcel Bonay,* Alain Grodet,* Vladimir Pelicic,† Brigitte Gicquel,† and Allan J. Hance2*

The mechanisms through which granuloma formation helps control mycobacterial infection are poorly understood, but it is possible that the accumulation of macrophages at high density at sites of infection promotes the differentiation of macrophages into cells with improved mycobactericidal activity. To test this possibility, varying numbers of monocytes were cultured in 96-well plates for 3 days, infected with Mycobacterium bovis bacillus Calmette-Guérin, and mycobacterial number was assessed 7 days after infection based on the measurement of luciferase activity expressed by a mycobacterial reporter strain or by counting CFU. Mycobacterial growth was optimal in cultures containing $5 \times 10^4$ cells/well, but increasing the number of cells to $2 \times 10^5$ cells/well resulted in complete inhibition of mycobacterial growth. This effect could not be explained by differences in mycobacterial uptake, multiplicity of infection, acidification of the extracellular medium in high density cultures, enhanced NO production, or paracrine stimulation resulting from secretion of cytokines or other proteins. The morphology of cells cultured at high density was strikingly different from that of monocytes cultured at $5 \times 10^4$ cells/well, including the appearance of numerous giant cells. The bacteriostatic activity of monocyte-derived macrophages was also dependent on cell number, but fewer of these more mature cells were required to control mycobacterial growth. Thus, the ability of human macrophages to control mycobacterial infection in vitro is influenced by the density of cells present, findings that may help explain why the formation of granulomas in vivo appears to be a key event in the control of mycobacterial infections. The Journal of Immunology, 2001, 166: 6203–6211.

Pathogenic mycobacteria survive and proliferate within mononuclear phagocytes, cells capable of eliminating most internalized organisms. The ability of the host response to increase the mycobactericidal activity of macrophages is clearly important in the development of an effective response. Numerous in vivo studies have shown that lymphocytes play an essential role in increasing the mycobactericidal activity of macrophages. For example, the depletion of CD4 T cells in humans infected with HIV-1 and the depletion of either CD4 or CD8 T cells in murine models leads to a severe impairment in the control of mycobacterial growth (1–4). A number of cytokines are produced by T cells in the course of responses to mycobacterial infection, and several of these mediators have been shown to be essential for the development of mycobactericidal activity in vivo. Thus, inactivation of genes coding for IFN-γ or its receptor, TNF-α or TNF receptors, IL-12, and IL-18 have all been shown to seriously impair host immunity in murine models (5–11), and the identification of mutations in the genes coding for IFN-γ and IL-12 receptors in individuals with increased sensitivity to mycobacterial infection have confirmed the absolute requirement for these cytokines in the control of mycobacterial infection in humans (12–15).

The mechanisms through which T cells increase the mycobactericidal activity of macrophages remain controversial. Studies evaluating murine macrophages in vitro have shown that cytokines produced by T cells, in particular the combination of TNF-α and IFN-γ, can directly stimulate macrophage bactericidal activity, at least in part through the induction of NO synthetase activity (16–18). Numerous attempts to demonstrate similar effects of these cytokines on human macrophages have failed. Whether used individually or in combination, TNF-α, IFN-γ, and IL-12 have not been found to reproducibly improve the mycobactericidal activity of human macrophages (19–22). Taken together, these findings suggest that exposure to T cell-derived cytokines alone is not sufficient for the development of potent mycobactericidal activity in human macrophages. Several recent studies have suggested that cell-cell interactions between T cells and macrophages may also contribute to the development of mycobactericidal activity (22–24).

In addition, lymphocytes may act indirectly, by fostering the formation of well-developed immune granulomas, which are a constant feature of effective antimycobacterial responses in both experimental models and humans. Granulomas are composed of a central core of monocyte-derived cells expressing characteristic morphologic features (epithelioid macrophages and giant cells) that is surrounded by and infiltrated with activated CD4+ and CD8+ T cells. Cytokine secretion by T cells is required for granuloma formation in vivo, because depletion of T cells or inactivation of signaling by IFN-γ, TNF-α, and IL-12 have all been shown to impair and/or delay granuloma formation (3–10, 25, 26), and the extent of these abnormalities correlates with the overall impairment in mycobactericidal activity observed.

The mechanisms through which granuloma formation helps control mycobacterial infection are poorly understood, but it is possible that the accumulation of macrophages at high density at sites of infection promotes the differentiation of macrophages into
cells with improved mycobacterial activity. To test this possibility, we have evaluated the effect of macrophage density and maturation on the intracellular growth of Mycobacterium bovis bacillus Calmette-Guérin (BCG),3 and demonstrate that culture at high density considerably improves the ability of human macrophages to control mycobacterial proliferation. The evaluation of the mechanisms involved suggests that differences in their differentiated state, not the production of autocrine/paracrine mediators, are chiefly responsible for the improved bacteriostatic activity of these cells.

Materials and Methods

Purification and culture of human monocytes

PBMCs were isolated from leukapheresis concentrates obtained from healthy volunteers by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). Monocytes were then purified by counterflow centrifugal elutriation (27), using a 12-21 ME centrifuge and a JE-6B rotor (Beckman Instruments, Palo Alto, CA). Monocytes had a viability of >95% and a purity of at least 92% in all experiments. For some experiments, monocytes that had been frozen at −80°C in 10% DMSO were used.

To initiate experiments, monocytes were resuspended in complete medium (IMDM (Sigma, St. Louis, MO) supplemented with 2 mM l-glutamine (Life Technologies, Gaithersburg, MD), 200 U/ml penicillin G, 1 μg/ml kanamycin (Sigma), and 20% human AB serum (Institut Jacques Boy, Reims, France)). Cells were cultured in 96-well flat-bottom plates, with opaque sides and transparent bottoms (EG&G Wallac, Turku, Finland) at concentrations ranging from 1 × 10^5 cells/well to a final volume of 200 μl medium. In most experiments, cells were maintained at 37°C in 95% air, 5% CO_2 for 3 days before infection. In some experiments, cells were cultured for 10 days before infection, in which case medium was replaced after 7 days of culture.

Infection of monocytes

The construction of the mycobacterial reporter strain used in these studies has previously been described (19). Briefly, to obtain this strain, M. bovis BCG 1173 P2 (Pasteur Institute, Paris, France) was transformed with the plasmid pPV12, which contains the mycobacterial origin of replication from pAL3000, a kanamycin resistance gene, and the luciferase gene Luc1 from Phothinus pyralis under control of the mycobacterial promoter Pan. Mycobacteria were grown at 37°C with gentle shaking in 7H9 liquid medium supplemented with Middlebrook ADC enrichment mixture (Difco, Detroit, MI) and 20 μg/ml kanamycin to an OD_650 of ~1, resuspended by pipetting, diluted with an equal volume of 40% glycerol, and stored as 1.2-ml aliquots at −80°C. On the day of infection, stocks were thawed, 1.2 ml complete medium without serum were added, and aggregates were dispersed by three cycles of sonication (50 W, 30 s, 0°C) using a Vibrachell sonicator (Sonic and Materials, Danbury, CT) followed by vortexing (5 s). Mycobacteria were pelleted by centrifugation (1800 × g for 10 min), resuspended in 3 ml complete medium without serum, and centrifuged (400 × g for 5 min) to pellet residual aggregates. The top 2.5 ml of the suspension were removed, and mycobacteria were quantified using a disposable count chamber (Kova Slide 10; Hycor Biomedical, Irvine, CA). In most experiments, mycobacteria were diluted to 2 × 10^3 mycobacteria/well. After a 30-min incubation, a 1/1000 dilution of E. coli B 96P luminometer, 1.6 s after automatic injection of 100 μl of the substrate solution containing luciferin and ATP (Luciferase Assay Reagent; Promega). In all experiments, results for each experimental condition, expressed as relative light units (RLU) (3)/well, are the means of triplicate determinations.

Evaluation of mycobacterial growth

Determination of luciferase activity. Mycobacterial number was assessed by measuring luciferase activity produced by the mycobacterial reporter strain as previously described (19). Briefly, 96-well plates where thawed, and 75 μl lysis reagent (Promega, Madison, WI) were added to lyse the macrophages and permeabilize the mycobacteria. Luminescence was then measured during a 50-s reading during a EGG/B G Berthold MicroLumat LB-96P luminometer, 1.6 s after automatic injection of 100 μl of the substrate solution containing luciferin and ATP (Luciferase Assay Reagent; Promega). In all experiments, results for each experimental condition, expressed as relative light units (RLU) (3)/well, were the means of triplicate determinations.

CFU. After removal of 175 μl medium from each well, 50 μl 7H9 Middlebrook medium containing 0.1% SDS and 20 μg/ml kanamycin (37°C) were added. After a 30-min incubation, 3 ml complete medium was added, and CFU were plated on petri dishes containing 7H10 medium supplemented with Middlebrook OADC enrichment mixture (Dilco) and 20 μg/ml kanamycin. Plates were incubated at 37°C for 3 wk before colonies were counted. Results are expressed as CFU/well, and in all experiments are the average of triplicate values for each experimental condition.

When mycobacterial number was evaluated in parallel 24 h after infection based on CFU and RLU, a CFU:RLU ratio of 9.2 ± 3.7 (n = 8) was observed; this ratio was not different for cultures containing 5 × 10^4 and 2 × 10^5 cells/well. As indicated above, cultures were infected with ~5 × 10^4 mycobacteria/well. As expected, luciferase activity 24 h after infection was ~5000 RLU/well in all experiments, and the activity was similar in cultures containing 5 × 10^4 and 2 × 10^5 cells/well.

Evaluation of morphology and cell number

Cytologic evaluation was performed on cells grown under conditions identical to those used to evaluate mycobacterial growth. To evaluate cell morphology and viability, medium was removed and replaced with solution containing 50 mg/ml propidium iodide and 3 μg/ml acridine orange in PBS, and cultures were examined under epifluorescence illumination using an inverted Zeiss fluorescent microscope. To identify mycobacteria, culture medium was removed, and cells were fixed with 10% formalin for 30 min, washed with 100 μl PBS, and stained for 20 min with 30 μg/ml acridine orange and 0.7 μg/ml propidium iodide in PBS. After washing with PBS, the bottom of each well was removed using a hollow steel punch, and the disc was attached to a microscope slide using Super Glue-3. Slides were immersed in methanol, dried, restained with 25 μl of the fluorescent dye solution, and rinsed successively with PBS and methanol. The slides were examined under epifluorescence illumination using a Leitz DMRD microscope camera system (Leica, Wetzlar, Germany). DNA content of cultures was determined using the method of Labarca and Pagen (28).

Modification of culture medium

In some experiments, the pH of culture medium (initially pH 7.7) was titrated with 1 M HCl to pH values between 6.4 and 7.5 after complete equilibration with 5% CO_2. To produce macrophage-conditioned medium, purified human macrophages were cultured in 96-well plates at 2 × 10^4 cells/well in 200 μl complete medium. After 7 days, the medium was removed, centrifuged at 600 × g for 10 min to remove residual cells, titrated to pH 7.5 by the addition of 1 N NaOH, filtered through a 0.22-μm pore size membrane (Millipore, Bedford, MA), and stored at 20°C. Culture medium conditioned by Jurkat cells (TIB-152; American Type Culture Collection (ATCC)) and A549 lung carcinoma cells (CCL-185; ATCC) was produced by similar techniques. Cell number was adjusted such that the pH of the conditioned medium after 7 days of culture was similar to that of macrophage-conditioned medium (pH 6.5–6.6). In some experiments, conditioned medium was evaluated after dialysis (Slide-A-Lyzer, Pierce, Rockford, IL) against complete medium without serum, after passage through filtration membranes with 3 or 100 kDa cutoffs (Centrisep concentrator; Amicon, Beverly, MA), in which case both the filtrate and the 10-fold concentrated retentate were tested, or after passage through a 1-ml C_18 hydrophobic chromatographic cartridge (Waters, Milford, MA). To evaluate the effect of N-iminomethylornithine hydrochloride (l-NIL; Cayman Chemical, Ann Arbor, MI) on myobacterial number at 37°C, 100 μl solution containing 4 μg/ml was prepared, and aliquots were added to the cultures to produce a final concentration of 5–125 μg/ml. Solutions were prepared contemporaneously from anhydrous solid at the time of addition.

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3 Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; l-NIL, N-iminomethylornithine hydrochloride; MOI, multiplicity of infection; RLU, relative light units.
Monocytes were cultured for 3 days in 96-well plates, infected with a suspension of M. bovis BCG, and mycobacterial number was assessed 7 days after infection based on the measurement of luciferase activity expressed by the mycobacterial reporter strain. Mycobacterial growth was highly dependent on the number of human monocytes present in the cultures (Fig. 1A). In the absence of monocytes, little mycobacterial growth was observed. In cultures containing $<5 \times 10^3$ macrophages/well, the viability of the heavily infected cells was poor. Thus, most mycobacteria were extracellular, because either they were deposited in the spaces between cells or released from dying cells, and no significant growth was seen. Mycobacterial growth was first observed in cultures containing $1 \times 10^4$ monocytes, and increased progressively as monocyte number was increased. Maximal mycobacterial growth was always observed in cultures containing either $2 \times 10^4$ ($n = 3$) or $5 \times 10^4$ cells/well ($n = 5$) in the eight experiments in which these two concentrations were compared. In cultures containing more monocytes, however, mycobacterial growth was partially ($1 \times 10^5$ cells/well) or almost totally suppressed ($2 \times 10^5$ cells/well) in all experiments.

To further explore this phenomenon, the kinetics of mycobacterial growth was evaluated in cultures containing numbers of macrophages that were optimal ($5 \times 10^5$ cells/well) or inhibitory ($2 \times 10^5$ cells/well) for growth (Fig. 1B). In cultures containing $5 \times 10^5$ cells/well, progressive mycobacterial growth was observed, resulting in a 15- to 5-fold increase in RLU between 1 and 7 days. For cultures containing $2 \times 10^5$ cells/well, modest mycobacterial growth was observed at day 4 (2.5- to 0.7-fold increase in RLU). Between days 4 and 7, however, luciferase activity decreased in six of nine experiments, resulting in an overall 1.4- to 0.4-fold increase in RLU between 1 and 7 days. When mycobacteria were cultured in the absence of macrophages, no net growth was seen at 7 days.

To confirm the important effect of macrophage density on mycobacterial growth, a second technique was used to evaluate mycobacterial number. When mycobacterial growth in cultures containing $5 \times 10^4$ and $2 \times 10^5$ cells/well was assessed by the measurement of CFU, the results were comparable to those obtained using the luciferase assay (Fig. 2). When mycobacteria were cultured in the absence of macrophages, the number of CFU decreased progressively in all four experiments (data not shown).

FIGURE 1. Effect of cell number on the growth of M. bovis BCG in cultured human macrophages. A, Monocytes were purified by elutriation, cultured at the indicated number per well for 3 days, and infected with M. bovis BCG. Seven days after infection, culture medium was removed and the luciferase activity (RLU) expressed by the mycobacterial reporter strain was measured by luminometry. Dashed line, RLU/well for cultures containing BCG only evaluated 1 day after infection. Results, expressed as RLU, are the mean ± SD for triplicate determinations from a one of two experiments that gave similar results. B, Cultures containing $5 \times 10^4$ cells/well (○), $2 \times 10^5$ cells/well (■), prepared as described above, or no monocytes (□) were infected with M. bovis BCG reporter strain, and luciferase activity was measured after the indicated time in culture. Results are the mean ± SD for nine independent experiments performed using cells obtained from different individuals.

FIGURE 2. Comparison of mycobacterial growth as assessed by measurement of luciferase activity and enumeration of CFU. Cultures containing $5 \times 10^4$ macrophages/well (○, ■) or $2 \times 10^5$ macrophages/well (□, □), prepared as described above, were infected with the M. bovis BCG reporter strain. After the indicated time in culture, the number of mycobacteria present were assessed by measuring luciferase activity (RLU, □). CFU and colony forming units (CFU, ○, ●). Results are presented as the mean ± SEM for four independent experiments performed using monocytes from different individuals.
well was increased 4-fold compared with that used to infect cultures containing $5 \times 10^5$ cells/well, resulting in the same MOI for these cultures, high density cultures, but not low density cultures, could still control mycobacterial growth (compare solid bars in Fig. 3). Similarly, when the number of mycobacteria used to infect cells containing $5 \times 10^5$ cells/well was decreased 4-fold compared with that used to infect cultures containing $2 \times 10^5$ cells/well, low density cultures were still permissive for mycobacterial growth (compare hatched bars in Fig. 3), again indicating that the effect of cell number on mycobacterial growth is independent of MOI.

Effect of extracellular pH on mycobacterial growth in cultured macrophages

Macrophages cultured at $2 \times 10^5$ cells/well progressively acidified the culture medium, an effect that occurred to a much lesser extent when cells were cultured at $5 \times 10^5$ cells/well (Fig. 4A). To evaluate whether such acidification of the extracellular medium could contribute to the ability of macrophages to control mycobacterial proliferation, cultures containing $5 \times 10^5$ cells/well were infected for 24 h, after which the culture medium was replaced with medium previously adjusted to pH values between 6.8 and 7.7. For cells cultured initially at pH 6.8, the pH after 7 days was as low as that observed in cultures containing $2 \times 10^5$ cells/well (Fig. 4B). Nevertheless, acidification of the extracellular medium had little impact on the growth of mycobacteria at 4 or 7 days in macrophages cultured at $5 \times 10^5$ cells/well, whereas mycobacterial growth was controlled in cultures containing $2 \times 10^5$ cells/well. Thus, changes in the pH of the extracellular medium could not account for the differences in mycobacterial growth in low and high density cultures.

Morphology and viability of cultured macrophages

When monocytes were plated at $5 \times 10^5$ cells/well, the cells rapidly formed a monolayer in which most cells were firmly attached to the surface. After infection, most cells remained adherent, although a small number of cell clusters were observed. The cells progressively increased in size, producing an essentially confluent monolayer at 7 days (Fig. 5A). At this time, essentially 100% of the cells were viable as assessed by their ability to exclude propidium iodide. After fixation and staining, small clusters of mycobacteria could be observed in most cells 7 days after infection (Fig. 5B). Although not quantified, the number of mycobacteria per cell clearly increased during the week after infection.

Several differences were observed when monocytes were plated at $2 \times 10^5$ cells/well. First, clusters of cells were prominent immediately after plating and increased in size after infection. Cell fusion, resulting in giant cell formation, was evident 4 days after infection; by 7 days after infection, large numbers of giant cells containing 5–15 nuclei were present (Fig. 5C). Despite the low pH of the extracellular medium, >90% of cells in high density cultures remained viable after 7 days of culture, although small numbers of dead cells were always present (orange nuclei in Fig. 5C). Subsequently, the viability of high density cultures declined, and at 10 days numerous dead cells were present. Cells with condensed nuclei typical of apoptotic cells were observed but were never...
abundant; very few nonadherent cells were observed in these cultures. To evaluate the possibility that dead cells were being lost from the cultures through ingestion and degradation by the remaining viable macrophages, the DNA content of adherent cells was measured after various times in culture. No significant change in DNA content was observed over the 7-day period in cultures containing 2 × 10^5 cells/well (day 0, 1.5 ± 0.5; day 7, 1.3 ± 0.6 μg DNA/well; n = 4; p > 0.05 by paired t test).

After fixation, mycobacteria were observed in high density cultures. In particular, individual mycobacteria or small aggregates were observed in the perinuclear region of some, but not all, giant cells (Fig. 5D, arrows). Isolated macrophages containing aggregates of mycobacteria, as observed in cultures plated at 5 × 10^4 cells/well, were infrequent. Overall, the number of mycobacteria per high power field was clearly reduced in high density cultures. The visualization of mycobacteria required fixation of the cells, and the viability of the intracellular mycobacteria could not be assessed.

**Effect of macrophage-conditioned medium on mycobacterial growth**

The impaired mycobacterial growth in high density cultures could result from either the production of factors by macrophages that improve their mycobactericidal activity or the depletion of metabolites necessary for mycobacterial growth. To test these possibilities, macrophages were cultured at 2 × 10^5 cells/well for 7 days, and the conditioned medium was removed, neutralized to pH 7.5, and added to cultures containing 5 × 10^4 macrophages/well 1 day after infection. As shown in Fig. 6A, mycobacterial growth at 7 days was significantly reduced in cells maintained in the presence of macrophage-conditioned medium at dilutions of 1/2 and 1/4 (p < 0.01 and p < 0.05, respectively) compared with that observed in cells cultured in unconditioned medium. The inhibitory activities of conditioned medium from infected and uninfected macrophages were similar (data not shown). Although mycobacterial growth was reduced in cells cultured with macrophage-conditioned medium for 7 days, significant proliferation did occur and was always greater than that observed in cultures containing macrophages at high density (percent inhibition of RLU relative to cultures containing 5 × 10^4 macrophages: 5 × 10^4 macrophages receiving conditioned medium on day 1, 68 ± 9%; 2 × 10^5 macrophages, 93 ± 2%; n = 5, p < 0.01). The effect observed with conditioned medium was not increased by addition on two occasions (percent inhibition of RLU: 5 × 10^4 macrophages receiving conditioned medium on days 1 and 4, 62 ± 26%; 2 × 10^4 macrophages, 93 ± 3%; n = 2). Conditioned medium did not influence the growth of mycobacteria cultured in the absence of monocytes at 4 or 7 days (p > 0.4 for both comparisons, n = 3).

Filtration of conditioned medium using filters with 100- or 3-kDa cutoffs did not decrease the inhibitory activity, although the activity was lost after dialysis (Table I). Heating of conditioned medium or passage through a C18 hydrophobic chromatography column did not reduce the inhibitory activity. The use of fresh medium that had been titrated to pH 6.5 using dilute HCl and then neutralized to pH 7.4 did not reduce mycobacterial growth.

When 5 × 10^4 macrophages were cultured in the presence of conditioned medium, their morphologic features were generally similar to those of cells maintained in standard medium. At 7 days, cell contours were somewhat less uniform and appeared to result from partial detachment of some cells from the surface. The prominent cluster formation and cell fusion seen in high density cultures was not observed.

To evaluate whether the suppressive effect on mycobacterial growth was a unique property of macrophage-conditioned medium, medium conditioned by other cell types was also evaluated. Jurkat cells and A549 carcinoma cells were grown in complete medium until the pH was equivalent to that of macrophage-conditioned medium, the medium was removed and neutralized to pH 7.5, and the effects of these conditioned media were compared (Fig. 6B). Although macrophage-conditioned medium was somewhat more inhibitory, medium conditioned by the other cell types also decreased mycobacterial growth at 7 days, and the differences in inhibition comparing the three media were not significantly different (n = 4, p > 0.05). As above, high density macrophages had a significantly greater inhibitory effect on mycobacterial growth than low density macrophages cultured with conditioned medium.
and 5 × 10^4 cells/well, but not cells plated at 2 × 10^5 cells/well, were permissive for mycobacterial growth (Fig. 7A). Monocytes cultured for 10 days before infection exhibited progressive differentiation into large macrophage-like cells that were firmly adherent to the culture wells. Interestingly, when cultured at 2 × 10^4 cells/well, these macrophage-like cells were still permissive for mycobacterial growth, but cultures containing 5 × 10^4 differentiated macrophages controlled mycobacterial growth as well as cultures containing 2 × 10^5 monocytes infected 3 days after isolation (p > 0.2 at 4 and 7 days comparing the two populations shown in Fig. 7, A and B).

**Discussion**

This study demonstrates that the ability of human monocyte-derived macrophages to control mycobacterial infection in vitro is highly dependent on the density at which these cells are present.

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### Table 1. Effect of pretreatment of macrophage-conditioned medium on its ability to inhibit mycobacterial growth in human macrophages

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>% Inhibition of Mycobacterial Growth</th>
<th>n</th>
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<tbody>
<tr>
<td>None</td>
<td>72.6 ± 11.8</td>
<td>7</td>
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<tr>
<td>100-kDa filtration</td>
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<td>Filtrate</td>
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<tr>
<td>Retentate</td>
<td>82.8 ± 2.6</td>
<td>2</td>
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<tr>
<td>3-kDa filtration</td>
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<tr>
<td>Filtrate</td>
<td>68.8 ± 20.8</td>
<td>2</td>
</tr>
<tr>
<td>Retentate</td>
<td>84.7 ± 2.1</td>
<td>2</td>
</tr>
<tr>
<td>Dialysis</td>
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<tr>
<td>Heating (95°C for 15 min)</td>
<td>15.8 ± 12.4</td>
<td>4</td>
</tr>
<tr>
<td>Hydrophobic chromatography (C&lt;sub&gt;18&lt;/sub&gt;)</td>
<td>77.1 ± 6.2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Conditioned medium was obtained from macrophages cultivated for 7 days at 2 × 10^5 macrophages/well, 1 day after infection with the M. bovis BCG reporter strain. Results are expressed as mean ± SD for the percent inhibition of mycobacterial growth compared with that of infected cultures not treated with conditioned medium.

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**Effect of L-NIL on mycobacterial growth in macrophages cultured at high density**

To evaluate whether NO production was required for the improved mycobacteriostatic activity of macrophages cultured at high density, the effect of the L-NIL, an inducible NO synthase inhibitor, was evaluated. The addition of 5 μg/ml L-NIL on the day of infection did not increase mycobacterial growth at 7 days (RLU/well at 7 days: control, 3870 ± 1326; L-NIL, 4140 ± 1878 RLU/well; p > 0.2). Increasing the concentration of L-NIL to 125 μg/ml or adding 5 μg/ml L-NIL every 48 h after infection also failed to increase mycobacterial growth (data not shown).

**Effect of macrophage maturity on the control of mycobacterial growth**

In the experiments described thus far, monocytes were infected after 3 days of culture in vitro. To determine whether the maturity of these cells influenced the relationship between cell number and their ability to control mycobacterial growth, monocytes from the same individuals cultured for 3 and 10 days before infection were compared. As described above, monocytes plated at 2 × 10^5 cells/well, these macrophage-like cells were still permissive for mycobacterial growth, but cultures containing 5 × 10^4 differentiated macrophages controlled mycobacterial growth as well as cultures containing 2 × 10^5 monocytes infected 3 days after isolation (p > 0.2 at 4 and 7 days comparing the two populations shown in Fig. 7, A and B).
When plated at a density that results in a single monolayer of cells after 7 days of culture, we found that monocytes were permissive for growth of *M. bovis* BCG, findings consistent with previous reports (29, 30). When the same cells were plated at a 2- to 4-fold higher density, however, mycobacterial growth was strongly inhibited, and, in some experiments net mycobactericidal activity was observed. These findings demonstrate that human monocytes have the intrinsic capacity to develop in vitro into cells capable of controlling mycobacterial infection.

The role of a number of possible mechanisms through which changes in cell density could influence mycobactericidal activity were investigated. Cell density did not change mycobacterial uptake by the cultured macrophages. Furthermore, the effect of macrophage density on mycobacterial growth was insensitive to the multiplicity of infection over a 16-fold range. Thus, neither of these factors could explain the observed effect.

The control of intraphagosomal pH is also thought to be an important determinant of mycobacterial survival in macrophages. Mycobacterial growth can be directly influenced by an acidic environment, and acidification can increase their sensitivity to oxidant-mediated killing (31, 32). Virulent mycobacteria have been reported to inhibit the acidification of phagosomes through exclusion of vesicular proton-ATPase complexes (33). Acidification of the extracellular milieu can also decrease the intracellular pH of macrophages (34), and we found that macrophages cultured at high density did acidify the extracellular medium to a pH as low as 6.5 after 7 days. Several observations suggested, however, that this change did not account for improved mycobacteriocidality. First, despite acidification of the culture medium to similar levels, macrophages cultured at lower density remained permissive for mycobacterial growth. In addition, the viability of high density macrophages remained excellent despite the low extracellular pH, suggesting that these cells maintained a physiologic intracellular pH via energy-dependent transport mechanisms. Similarly, the bacteriocidal activity of macrophages cultured at high density could not be attributed to the preferential generation of NO by these cells, because treatment with the inducible NO synthase inhibitor L-NIL did not influence their ability to control mycobacterial replication. Consistent with these observations, prior studies have shown that although human macrophages express inducible NO synthase after mycobacterial infection (35, 36), the levels of NO produced are low, and the inhibition of NO production by human macrophages does not reproducibly increase mycobacterial growth (36, 37). Increased apoptosis of macrophages infected with mycobacteria has been described and in some but not all cases has been associated with mycobactericidal killing (38–41). In this study, the viability of macrophages cultured at high density was excellent at 7 days, few detached cells were present, and the DNA content of the cultures was stable during the 7-day culture period. These findings indicate that macrophage apoptosis or ingestion of infected apoptotic cells by neighboring uninfected macrophages is unlikely to account for the reduced mycobacterial growth seen in high density cultures.

Two observations in our study suggested that the differentiated state of macrophages have an important bearing on their ability to control mycobacterial infection and that both the time in culture and the density of cells present could influence this differentiation process. The mycobacteriostatic activity of both freshly isolated monocytes and macrophages that had been cultured for 10 days before infection was dependent on the number of cells present in the cultures, but fewer of the more differentiated macrophage-like cells were required to control mycobacterial proliferation. Prior studies have shown that the maturation in vitro of human monocytes before infection can modify their capacity to control mycobacterial replication. In these studies, however, an increase (42), a progressive decrease (43), or an initial decrease followed by an increase (44) in the permissiveness for mycobacterial infection has been observed as a function of the time in culture. The important effect of cell density was not evaluated in these studies and may account, at least in part, for the discrepant findings. We also observed that the morphologic features of monocytes cultured at high density were strikingly different from those of cells cultured at low density. In high density cultures, macrophages were larger, cell aggregates were prominent during the first few days of culture, and later times the progressive appearance of giant cells was observed. These changes occurred to a similar extent in uninfected cultures, indicating that mycobacterial infection was not responsible. Byrd (45) previously observed an association between giant cell formation and the ability of cultured monocytes to inhibit mycobacterial proliferation. As in his study, we observed that mycobacteria accumulated in the perinuclear area of giant cells, which may restrict the invasion of adjacent cells (45). A number of signals have been described that increase giant cell formation in cultured macrophages (46–48). Conditioned medium from high density macrophage cultures did not induce giant cell formation in low density cultures, suggesting that paracrine stimulation by cytokines was not sufficient for the induction of giant cell formation seen in these studies. Culture of cells at high density resulted in the early formation of cell aggregates, compatible with the idea that prolonged membrane contact may have been important.

It should be emphasized that the relationship between increased mycobacteriostatic activity and macrophage differentiation observed in these studies does not prove that the processes are causally related. Furthermore, giant cell formation was not a feature of either low density cultures treated with conditioned medium or macrophages cultured at 5 × 10^6 cells/well for 10 days before infection, although these populations were able, respectively, to partially or completely inhibit mycobacterial replication. Thus, this type of differentiation, although possibly important, was not indispensable for improved mycobacteriostatic activity. Future studies evaluating the effect of inhibiting macrophage differentiation on mycobacteriostatic activity may prove informative. In this regard, we have recently found that when monocytes are cultured with type I IFNs, they persist as small “monocyte-like” cells for up to 7 days. Under these conditions, monocytes are very permissive to mycobacterial growth, even when cultured at high density (F. Bouchonnet, N. Boechat, M. Bonay, M. Vokurka, and A. J. Hance, manuscript in preparation).

An interesting finding in the current studies was the observation that when low density macrophages were maintained in the presence of medium conditioned by high density macrophages, intracellular mycobacterial growth was reduced. This finding is compatible with the possibility that high density cultures release factor(s) that can stimulate mycobacteriostatic activity or deplete metabolites necessary for mycobacterial growth. The observation that medium conditioned by other cell types also decreased mycobacterial growth in low density macrophages suggests that this effect is not specific for macrophages. Furthermore, modification of the extracellular milieu did not appear to completely explain the increased mycobacteriostatic activity of cells cultured at high density, because low density macrophages cultured in conditioned medium inhibited mycobacterial growth less well than macrophages cultured at high density, and cells cultured in conditioned medium, unlike high density cultures, did not inhibit mycobacterial growth at 4 days.

The ability of conditioned medium to enhance mycobacteriostatic activity was not decreased by heating, passage through a membrane with a 3-kDa cutoff or passage through a C16 hydrophobic
column, whereas the activity was completely lost by dialysis. In addition, the 10-fold concentrated retentate obtained by ultrafiltration of conditioned medium did not contain increased activity. These findings argue strongly against a role for soluble secreted proteins or lipid mediators in this phenomenon. Thus, although cultured macrophages are secretive large quantities of cytokines, including IFN-γ, TNF-α, and IL-12 (22, 23, 30, 49, 50), our results are compatible with prior studies indicating that these cytokines are not sufficient to induce strong mycobactericidal activity in otherwise permissive human macrophages. Low molecular mass substances produced by macrophages, and other cell types can modify the activity of macrophages and improve their resistance to intracellular pathogens, (e.g., ATP and nonhexeme iron) (20, 51), and the release of such agents could explain our findings. Attempts to isolate a putative stimulatory factor by ion exchange chromatography were unsuccessful, but the buffers required for these studies proved to be toxic for macrophages, which complicated this endeavor (data not shown). Alternatively, it is the possibility that substances that deactivate macrophages or that are required for optimal mycobacterial growth had been depleted by the metabolically active cells (e.g., iron, adenosine, amino acids) (52–56). The activity of conditioned medium was completely lost after dialysis against serum-free medium, indicating that the culture medium, not human serum, would have to be the source of such a metabolite. Furthermore, conditioned medium diluted 4-fold with fresh medium retained some activity, this metabolite would have to be present in suboptimal amounts in fresh medium. Further studies will be required to identify the substances responsible for the effects observed with conditioned medium and to determine whether macrophages within granulomas can “condition” their environment in a similar fashion.

Taken together, these results indicate that the ability of human macrophages to control mycobacterial infection in vitro is strongly influenced by the density of cells present and indicate that cell density in the culture in which before culture infection can influence the differentiated state of these cells and their capacity to modify the external milieu. The requirement for both monocytes and more mature macrophages to be present at a critical density to suppress mycobacterial proliferation offers insights into why the formation of granulomas in vivo appears to be a key event in the control of mycobacterial infections. The availability of this model, in which the same cells are either permissive or resistant to mycobacterial infection, should be useful in further characterizing the cellular events that are indispensable for the development of mycobactericidal activity.

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