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An In Vitro Model for the Lepromatous Leprosy Granuloma: Fate of Mycobacterium leprae from Target Macrophages after Interaction with Normal and Activated Effector Macrophages

Deanna A. Hagge, Nashone A. Ray, James L. Krahenbuhl, and Linda B. Adams

The lepromatous leprosy granuloma is a dynamic entity requiring a steady influx of macrophages (Mφ) for its maintenance. We have developed an in vitro model to study the fate of Mycobacterium leprae in a LL lesion, with and without immunotherapeutic intervention. Target cells, consisting of granuloma Mφ harvested from the footpads of M. leprae-infected athymic nu/nu mice, were cocultured with normal or IFN-γ-activated (ACT) effector Mφ. The bacilli were recovered and assessed for viability by radiorespirometry. M. leprae recovered from target Mφ possessed high metabolic activity, indicating a viable state in this uncultivable organism. M. leprae recovered from target Mφ incubated with normal effector Mφ exhibited significantly higher metabolism. In contrast, bacilli recovered from target Mφ cocultured with ACT effector Mφ displayed a markedly decreased metabolic activity. Inhibition by ACT Mφ required an E:T ratio of at least 5:1, a coculture incubation period of 3–5 days, and the production of reactive nitrogen intermediates, but not reactive oxygen intermediates. Neither IFN-γ nor TNF-α were required during the cocultivation period. However, cell-to-cell contact between the target and effector Mφ was necessary for augmentation of M. leprae metabolism by normal effector Mφ as well as for inhibition of M. leprae by ACT effector Mφ. Conventional fluorescence microscopy and confocal fluorescence microscopy revealed that the bacilli from the target Mφ were acquired by the effector Mφ. Thus, the state of Mφ infiltrating the granuloma may markedly affect the viability of M. leprae residing in Mφ in the lepromatous lesion. The Journal of Immunology, 2004, 172: 7771–7779.

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2 Address correspondence and reprint requests to Dr. Linda B. Adams, Immunology Research Department, National Hansen’s Disease Programs, Laboratory Research Branch, Louisiana State University School of Veterinary Medicine, Skip Bertman Drive, Baton Rouge, LA 70803. E-mail address: ladams1@lsu.edu

3 Abbreviations used in this paper: LL, lepromatous leprosy; CMI, cell-mediated immunity; AFB, acid-fast bacillus; TT, tuberculoid leprosy; Mφ, macrophage; ACT, activated; RNI, reactive nitrogen intermediate; ROI, reactive oxygen intermediate; EGFP, enhanced green fluorescent protein; γ-NIL, γ-aminosuccinic acid; LSI, L-tryptophan; LAK, lymphokine-activated killer.

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Toward the tuberculoid end of the spectrum, Mφ function as potent effector cells of resistance and are responsible for killing and eliminating M. leprae. T cells generate the Mφ activation factor, IFN-γ, and if Mφ are activated (ACT) with IFN-γ before infection with M. leprae, they can efficiently kill the bacilli (10). However, there is evidence that M. leprae-infected Mφ, especially if heavily infected for a prolonged period of time, are refractory to activation by IFN-γ (11–13). This implies that killing of M. leprae in infected Mφ is likely accomplished by new ACT Mφ that migrate into the lesion in response to various chemotactic stimuli. How does the new ACT Mφ acquire and kill M. leprae? There is ample evidence that M. leprae-specific CTLs are generated at the tuberculoid end of the spectrum, and that these T cells lyse M. leprae-infected Mφ and Schwann cells (14–16). This is most likely the primary mechanism of cell turnover in TT. However, we wondered whether or not ACT Mφ play a role in cell turnover; there are precedents that suggest that they may. First, numerous tumor models have shown that Mφ can act as killer cells and attack abnormal cells (17–22). Second, immunotherapy with Th1-type cytokines in LL patients demonstrated upgrading of clinical disease in the absence of specific T cell-mediated immunity (23, 24).

Therefore, as part of our efforts to develop an in vitro model for the leprosy granuloma, we investigated the fate of M. leprae from infected target Mφ upon coculture with new Mφ. We examined whether new Mφ could acquire the bacilli from infected target Mφ, if the viability of the bacilli was modified, and what Mφ effector mechanisms were involved. We provide evidence that effector Mφ, both natural and ACT, acquired the bacilli from the target Mφ in a contact-dependent manner. Coculture of infected target Mφ with normal effector Mφ augmented M. leprae metabolic activity, whereas coculture with ACT effector Mφ decreased M. leprae metabolism via a reactive nitrogen intermediate (RNI)-dependent pathway.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Enhanced green fluorescent protein (EGFP)-expressing transgenic mice were originally obtained from The Jackson Laboratory and were bred in our vivarium. Inducible NO synthase knockout (NOS2−/−) mice (The Jackson Laboratory) and mice deficient in the phagocyte oxidase gp91 subunit (phox91−/−) (The Jackson Laboratory), as well as athymic nu/nu mice (The Jackson Laboratory), all on a B6 background, were housed under aseptic conditions in microisolators (Microisolator Mouse VCL, Exhaust Rack Housing System; Lab Products, Seaford, DE).

Cultivation and maintenance of M. leprae

M. leprae are maintained in the footpads of nu/nu mice by programmed passage to assure a weekly supply of fresh, highly viable bacilli (25).

Cell culture

Bone marrow-derived Mφ.

Bone marrow Mφ were cultured as previously described (26). Briefly, cells were harvested from both femurs and seeded onto 13-mm thermax coverslips (Nalge Nunc International, Na-perville, IL) in 24-well tissue culture plates (Corning, Corning, NY) in culture medium (DMEM containing HEPES and sodium bicarbonate (Life Technologies Invitrogen, Carlsbad, CA), 10% FBS (HyClone, Logan, UT), 2 mM l-glutamine (Life Technologies Invitrogen), and 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO)) supplemented with 5 μg/ml M-CSF (R&D Systems, Minneapolis, MN). After 6 days of incubation at 37°C, nonadherent cells were removed with vigorous washing in PBS (Irvine Scientific, Santa Ana, CA), and the adherent Mφ monolayer was incubated in culture medium without M-CSF for an additional day.

Resident peritoneal Mφ.

Resident peritoneal cells were harvested by lavage and cultured at 2 × 10⁶ cells per well on coverslips in culture medium overnight at 37°C, and the adherent Mφ population was purified by vigorous washing to remove nonadherent cells.

Footpad granuloma Mφ.

Granuloma Mφ were harvested from the footpads of athymic nu/nu mice, infected 9–14 mo previously with 1 × 10⁸ viable M. leprae using a modification of the method of Sibbey and Krahenbuhl (11). Granuloma tissue was aseptically removed from each footpad and carefully sliced into small fragments with scalpels. The tissue fragments were digested in 2 ml of RPMI 1640 (Life Technologies Invitrogen) containing 20% FBS and 0.7 mg/ml collagenase XI-S (Sigma-Aldrich) and 30 μg/ml DNase 1 (Sigma-Aldrich) by end-over-end rotation for 30 min at 37°C. The tissue digestes were incubated on ice for 5 min to allow the tissue fragments to settle, and the supernatants, which contained a single-cell suspension of granuloma cells, were collected. The digestion procedure was repeated three times. The supernatant from the first digest was generally discarded, because it often contained large amounts of tissue debris. The supernatants of the second through fourth digestes were pooled, and the granuloma cells were washed three times in culture medium by centrifugation at 200 × g for 10 min at 4°C. Remaining tissue debris in the cell suspension was removed by centrifugation over Ficoll-Paque PLUS (Pharmacia, Uppsala, Sweden). The granuloma Mφ were collected, washed three times in culture medium, and counted. These footpad granuloma Mφ are viable and functional cells (e.g., adherent, phagocytic, non-specific esterase positive, and support the growth of Toxoplasma gondii) (11). Furthermore, microscopic analyses of the cell suspensions and cell-free supernatants verified that 99% of the M. leprae are located intracellularly.

Effectortarget Mφ coculture experiments

Normal or ACT (500 U/ml recombinant murine IFN-γ (R&D Systems) and 5–10 ng/ml LPS (Sigma-Aldrich) for 24 h at 37°C) peritoneal or bone marrow-derived Mφ were overlaid with 5 × 10⁶ target Mφ (M. leprae-infected footpad granuloma Mφ). All effector/target Mφ cocultures were incubated at 37°C, which is the optimum temperature for maintaining M. leprae viability in vitro both axenically (25) and in cultured mammalian cells (27, 28). In experiments analyzing the requirement for cell-to-cell contact, target Mφ were placed in Transwell inserts (4-μm pore size; Corning) and placed over the effector Mφ cultured on coverslips in the wells of the 24-well plate.

Reagents

l-Ν,N′-(1-Minoethyl)lysine hydrochloride (l-NIL) and l-Ν,N′-monomethyl-l-arginine (l-NMA) were obtained from Sigma-Aldrich and ChemBiochem (Salt Lake City, UT) respectively. The concentration of nitrite in the culture supernatants was determined using the Griess reagent (29). Abs to control Ab (clone R3-34) were obtained from BD PharMingen (San Diego, CA). ELISA kits for the detection of TNF-α (clone XM1G1.2) and an isotype control Ab (clone R3-34) were obtained from BD Pharmingen (San Diego, CA). Abs to l-NIL and l-NMA were obtained from ChemBiochem (Salt Lake City, UT). Abs to TNF-α (clone MP6-XT3) and IFN-γ (clone XM21) and IL-2 in the culture supernatants were obtained from R&D Systems.

Determination of M. leprae viability

The viability of M. leprae recovered from the Mφ cultures was ascertained by radiorespirometry, which measures the oxidation of [14C]palmitic acid to 14CO2, as described previously (10). Briefly, adherent Mφ were lysed in 200 μl 0.25% SDS (Sigma-Aldrich) to release intracellular M. leprae. After addition of an equal volume of RPMI 1640 plus 20% FBS, 300 μl of lysate was transferred to a 6-ml screw-cap vial containing 4 ml of commercially prepared BACTEC7H12B medium (BD Biosciences, Mountain View, CA) plus 5 μg/ml ampicillin (Sigma-Aldrich) and 2.5 μg/ml amphotericin B (Sigma-Aldrich). The vials, with caps loosened, were placed in a waterbath at 37°C, and after 7 days, cumulative amounts of oxidized palmitic acid released as 14CO2 by metabolically active M. leprae were measured using a Beckman LS6000ic scintillation counter (Beckman Coulter, Fullerton, CA). Data obtained in this radiorespirometry assay show strong correlation with M. leprae viability as determined in the mouse footpad growth assay (25) and in viability staining assays.[4]


Histopathology

The feet from M. leprae-infected nu/nu mice were fixed in 10% buffered formalin, decalcified, and embedded in paraffin. Four-micrometer sections were prepared of cross-sections at the distal, mid, and proximal areas of the metatarsals of the infected foot. The sections were stained with H&E and Fite’s acid-fast stain.

Flow cytometry

The footpad granuloma cells were incubated with rat anti-human CD16/CD32 (BD Pharmingen) for 10 min at 4°C to block FcRs and then stained for cell surface markers for 30 min at room temperature. The cells were processed for acid-fast staining by fixation with 10% formaldehyde in ethanol and staining using the Defco BBL TB Stain kit (BD Biosciences) or processed for differential staining with Diff-Quik reagents (American Scientific Products). TB Stain kit (BD Biosciences) or processed for differential staining with Diff-Quik reagents (American Scientific Products, McGraw Park, IL). Images were captured on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using a Spot RT camera and software (Diagnostic Instruments, Sterling Heights, MI).

Light microscopy

A total of 1 × 10^6 granuloma MΦ was centrifuged onto a slide using a Cytospin 2 (Thermo Shandon, Pittsburgh, PA) at 140 × g for 5 min at room temperature. The cells were processed for acid-fast staining by fixation with 10% formaldehyde in ethanol and staining using the Defco BBL TB Stain kit (BD Biosciences) or processed for differential staining with Diff-Quik reagents (American Scientific Products, McGraw Park, IL). Images were captured on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using a Spot RT camera and software (Diagnostic Instruments, Sterling Heights, MI).

Fluorescence microscopy

Fluorescent effector MΦ. Fluorescent peritoneal MΦ were obtained from either EGFP mice or from B6 mice labeled using the PKH67 Green Fluorescent Cell Linker mini-kit (Sigma-Aldrich). Briefly, 5 × 10^6 peritoneal cells were stained and washed according to the manufacturer’s instructions. Peritoneal cells were plated at 2 × 10^5 cells per culture in 35-mm glass-bottom petri dishes (Electron Microscopy Sciences, Fort Washington, PA), and nonadherent cells were removed by washing after overnight incubation at 37°C.

Fluorescent M. leprae. Freshly harvested viable M. leprae were labeled using the PKH26 Red Fluorescent Cell Linker mini-kit (Sigma-Aldrich). Briefly, 4 × 10^8 bacteria were stained and washed according to the manufacturer’s instructions and resuspended in 0.2 ml of PBS. Staining with this dye has no detrimental effect on M. leprae viability as measured by radiospirometry and growth in the mouse footpad. Fluorescent PKH26-labeled M. leprae was inoculated into the granulomatous footpads of nu/nu mice 11 mo after initial infection with unlabeled viable M. leprae. The granuloma MΦ were harvested 1 wk later and used as target cells.

Microscopy. Target MΦ infected with PKH26-labeled M. leprae were cocultured with EGFP effector MΦ for 5 days. The cocultures were washed three times in PBS and mounted in 10% glycerol in PBS containing 2 μg/ml each of minocycline (Sigma-Aldrich), ofloxacin (Sigma-Aldrich), and rifampin (Sigma-Aldrich). Confocal images were obtained using a Nikon Eclipse TE-2000E confocal microscope (Nikon Instruments, Lewisville, TX). Fluorescent images were also captured using live targets containing PKH26-labeled M. leprae cocultured with EGFP- or PKH67-labeled effector MΦ for 5 days. These cultures were rinsed three times and placed in PBS before viewing with a Zeiss Axiovert 405M microscope (Zeiss) with a Spot RT camera and software. Images were superimposed using Adobe Photoshop (Adobe Systems, San Jose, CA).

Statistical analyses

All statistics were performed using unpaired t tests in GraphPad InStat Software, version 3.00 (GraphPad Software, San Diego, CA). Data were considered significant at p < 0.05.

Results

Footpad granuloma target MΦ

Inoculation of M. leprae into the footpads of athymic nu/nu mice resulted in an enlarged footpad (Fig. 1A) comprising a lepromatous-type lesion (A2). Using a series of gentle digestions with collagenase and DNase, we obtained an average of 1.49 × 10^6 granuloma MΦ (n = 5; range, 7.55 × 10^5 to 27.2 × 10^6, depending on the size of the footpad) (Fig. 1A3). These MΦ were engorged with M. leprae and contained an average of 120 ± 17.7 AFB per cell (Fig. 1A4). Flow cytometric analyses of these footpad cell preparations demonstrated that they were composed primarily of Mac-1+ cells (Fig. 1B4). CD3+ cells (Fig. 1B1), B220+ cells (B2), and NK cells (B3) constituted ~0.37, 0.21, and 0.05% of the population, respectively.

Titration of E:T ratio

To determine the concentration of MΦ for optimal effector and target cell interaction, M. leprae-infected footpad granuloma target MΦ were cultured alone or cocultured with normal or ACT MΦ at various E:T ratios. As shown in Fig. 2A, an E:T ratio of 1.25:1 or 2.5:1 was not sufficient for killing of M. leprae by ACT effector cells.
of target Mφ with normal Mφ enhanced \textit{M. leprae} metabolic activity.

\textit{Inhibition of intracellular M. leprae by ACT Mφ is independent of reactive oxygen intermediates (ROI) but dependent on RNI}

To determine the importance of RNI and ROI in effector and target cell interaction, \textit{M. leprae}-infected footpad granuloma target Mφ were cultured alone or cocultured with normal or ACT effector Mφ from B6 or phox91−/− KO mice in the presence or absence of L-NIL, an inhibitor of the \textit{l}-arginine-dependent production of RNI, or normal or ACT effector Mφ from NOS2−/− mice. The viability of \textit{M. leprae} as well as the levels of nitrite in the culture supernatants was assessed. As shown in Table I, \textit{M. leprae} recovered from target Mφ cultured alone exhibited high metabolic activity, and very low levels of nitrite were detected in the culture supernatants. Likewise, target Mφ cocultured with normal B6 effector Mφ contained low nitrite concentrations; however, \textit{M. leprae} metabolic activity was significantly higher in these cocultures. In contrast, high levels of nitrite were found in the supernatants from target Mφ cocultured with ACT B6 Mφ, which inversely correlated with the low \textit{M. leprae} metabolic activity in these cocultures. In the presence of L-NIL, nitrite production in the cultures of target Mφ cocultured with ACT effector Mφ was inhibited, and the metabolic activity of \textit{M. leprae} remained high. Incubation of cocultures of target Mφ and ACT effector Mφ in the presence of 500 μM L-NMA, another inhibitor of RNI production, also prevented killing of \textit{M. leprae} and nitrite production (data not shown). Results comparable with those obtained with B6-derived effector Mφ were obtained when \textit{M. leprae}-infected footpad granuloma target Mφ were cocultured with effector Mφ obtained from phox91−/− mice (Table I). The importance of reactive nitrogen products was further substantiated using \textit{M. leprae}-infected target Mφ cocultured with NOS2−/− effector Mφ. As shown in Table I, ACT NOS2−/− effector Mφ were incapable of generating high levels of nitrites and could not kill \textit{M. leprae} in target Mφ. In fact, both normal and ACT effector Mφ from this strain of mice enhanced \textit{M. leprae} metabolic activity.

\textit{Neither TNF-α nor IFN-γ are required during coculture}

To determine whether IFN-γ or TNF-α were required for effector Mφ to modify the metabolic activity of target Mφ-derived \textit{M. leprae}, the cocultures were incubated in the presence of Abs to these cytokines. As shown in Fig. 3A, \textit{M. leprae}-infected \textit{nu/nu} footpad granuloma target Mφ produced high levels of TNF-α, which was significantly reduced upon cocultivation with both normal and ACT effector Mφ. In the presence of anti-IFN-γ, the levels of TNF-α generated by target Mφ was reduced ~40%, but was still higher than that produced in the cocultures. Negligible amounts of TNF-α were seen in all cultures in the presence of anti-TNF-α. Conversely, low but measurable amounts of IFN-γ were seen in the cocultures, primarily of target Mφ and ACT effector Mφ (Fig. 3B), and the levels of this cytokine were reduced in the presence of anti-IFN-γ. However, the presence or absence of these Abs had no effect on the viability of \textit{M. leprae} or on the amount of nitrite generated compared with control Ab. In the presence of each Ab, cocultivation of \textit{M. leprae}-infected target Mφ with normal effector Mφ augmented \textit{M. leprae} metabolic activity, whereas cocultivation with ACT effector Mφ decreased bacterial metabolism (Fig. 3C); again, \textit{M. leprae} viability was inversely correlated to nitrite levels (D).

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Incubation of \textit{M. leprae}-infected \textit{nu/nu} footpad granuloma target Mφ with normal or ACT bone marrow-derived effector Mφ affects \textit{M. leprae} viability. A, Titration of E:T ratio. Target cells were cultured alone (□) or cocultured with various numbers of normal (■) or ACT (●) effector Mφ for 5 days at 33°C. The Mφ were lysed, and the viability of \textit{M. leprae} was determined by radioisopetry. Data shown are representative of three independent experiments. B, Time course of metabolic inhibition by ACT effector Mφ. Target cells were cultured alone (□) or cocultured with normal (■) or ACT (●) effector Mφ at an E:T ratio of 20:1 for various lengths of time at 33°C. The Mφ were lysed, and the viability of \textit{M. leprae} was determined by radioisopetry. Data shown are representative of two independent experiments. Results are means ± SD. *, \textit{p} < 0.05; **, \textit{p} < 0.01; ***, \textit{p} < 0.001; ****, \textit{p} < 0.0001.}
\end{figure}

Mφ. However, an E:T ratio of 5:1 or greater yielded strong inhibitory activity by ACT Mφ while \textit{M. leprae} metabolism in target Mφ cocultured with an equal number of normal effector Mφ remained high. In fact, coculture of target Mφ with normal effector Mφ at a ratio of at least 2.5:1 consistently yielded significantly higher metabolic activity in the recovered \textit{M. leprae}.

\textit{Time course of metabolic inhibition by ACT Mφ}

To determine the optimal incubation period for effector and target Mφ coculture, \textit{M. leprae}-infected footpad granuloma target Mφ were cultured alone or cocultured with normal or ACT Mφ at an E:T ratio of 20:1 for various lengths of time. As shown in Fig. 2B, the metabolic activity of \textit{M. leprae} harvested from the target-only cultures remained constant over the 5-day cultivation period. Coculture of target Mφ with ACT effector Mφ for up to 48 h resulted in little change in \textit{M. leprae} metabolic activity. However, 72 h of coculture yielded a significant reduction in \textit{M. leprae} metabolism that was further reduced after 5 days of coculture. Again, coculture
Cell-to-cell contact is required for inhibition of intracellular M. leprae by ACT MΦ

To determine whether cell-to-cell contact was required for optimum effector and target cell interactions, M. leprae-infected footpad granuloma target MΦ were cultured in Transwell inserts that were overlaid onto effector MΦ monolayers. As shown in Fig. 4, target MΦ cultured on Transwell inserts yielded M. leprae with high viability. When target MΦ were cultured over normal effector MΦ, M. leprae metabolism remained high; however, there was no augmentation of metabolic activity like when the target and effector MΦ were in close contact (Figs. 2 and 3C, Table I). M. leprae-infected target MΦ cultured over ACT effector MΦ also yielded viable M. leprae. This inability of the ACT effector MΦ to kill the target cell-derived M. leprae occurred even though elevated levels of nitrites were generated by these effector cells.

Fate of M. leprae after coculture of infected target MΦ with effector MΦ

Granuloma MΦ containing fluorescent PKH26-labeled M. leprae were isolated from the footpads and placed in culture as targets with either green PKH67-labeled normal (Fig. 5A) or ACT (B) effector MΦ or EGFP-derived normal (C) or ACT (D) effector MΦ and incubated for 5 days at 33°C. Imaging by conventional fluorescence microscopy with computer-assisted image overlay (Fig. 5, A and B) demonstrated that the target MΦ-derived bacilli were acquired by both the normal and ACT effector MΦ. These observations were confirmed using confocal microscopy. Images of a single confocal plane (Fig. 5, C and D) verify that the bacilli are inside the effector MΦ. As shown above, under these conditions, the metabolic activity of M. leprae was greatly inhibited in cocultures of infected target MΦ with ACT effector MΦ and bolstered when infected target MΦ were cocultured with normal effector MΦ.

Discussion

In the current report, we have addressed the interplay between MΦ heavily infected with M. leprae and uninfected MΦ in vitro. Thus, the relevance of these studies focuses on the lepromatous end of the immunohistological spectrum where the disease evolves slowly and is characterized by the gradual accumulation of enormous numbers of bacilli in a local environment that is relatively free of T cells in a host incapable of mounting a specific CMI to the leprosy bacillus. By developing an in vitro model to follow the fate of M. leprae in an LL lesion, we sought, first, to study the host cell (MΦ) dynamics required to maintain and advance the course of clinical disease and, second, to model immunotherapeutic intervention and determine whether ACT effector MΦ alone could interact with the infected target MΦ and have an adverse effect on the viability of the M. leprae residing therein.

Previously, we have shown that granuloma MΦ can be isolated from the M. leprae-infected footpads of the athymic nu/nu mouse (11, 12). We have now optimized this procedure such that we can routinely obtain large numbers of viable, mature, M. leprae-infected MΦ in a suspension that is virtually free of extracellular bacilli. Use of these granuloma MΦ as our target cells has three major advantages. First, these cells differentiated, matured, and became infected in vivo, and are collected directly from the microenvironment of a lepromatous-type lesion. These characteristics make them a highly relevant MΦ population for immunological studies in LL. Second, because the granuloma MΦ are from nu/nu mice and the effector cells are prepared as pure adherent MΦ, we have a system allowing investigation of MΦ interaction without contamination with potentially CTLs (30, 31). Third, to coculture target MΦ with effector MΦ, it is necessary to have at least one of these populations prepared as a suspension culture. Our gentle digestion procedure eliminates the widely used but undesirable and potentially membrane-damaging isolation step of scraping adherent target or effector MΦ from culture dishes to obtain MΦ in suspension. This model is thus appropriate for this initial study of the dynamics of the LL granuloma microenvironment.

Although these granuloma MΦ are heavily burdened with bacilli, they are viable and functional cells that display many characteristics of normal uninfected MΦ in that they are adherent, phagocytic, and express FcRs and the Mac1 Ag; are nonspecific esterase positive; support the growth of T. gondii (11); and, as shown in this manuscript, maintain the viability of M. leprae. However, these MΦ do have a key defect. Unlike peritoneal or

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### Table I. Regulation of M. leprae viability following effector/target cell interactions by RNI

<table>
<thead>
<tr>
<th>Coculturesa</th>
<th>L-NIL</th>
<th>Cumulative 14CO2 (cpm (10^-1))</th>
<th>Nitrite (μM)</th>
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<td>B6 T-MΦ only</td>
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<td>1.6 ± 0.1</td>
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aM. leprae-infected mnu/mnu footpad granuloma target MΦ (T-MΦ) were cultured alone or cocultured with normal or ACT bone marrow-derived effector MΦ (E-MΦ) at an E:T ratio of 20:1 for 5 days at 33°C in the presence or absence of L-NIL. The culture supernatants were tested for nitrite production using the Griess reagent. The MΦ were lysed, and the viability of M. leprae was determined by radiorepirometry. Data shown are representative of three independent experiments. Results are means ± SD.

b<0.01.

c<0.05.

d<0.001.

### References

(11); and, as...
bone marrow-derived Mφ, which will phagocytize and kill reasonable numbers of *M. leprae* if ACT with IFN-γ (29, 32), the *M. leprae*-engorged granuloma Mφ are refractory to activation by IFN-γ and thus manifest aberrant effector functions, including impaired microbialcidal and tumoricidal capacity, decreased oxidative metabolic state, and lowered MHC class II Ag expression (11, 12). This defect appears to be the consequence of long-term infection with a high bacillary load because resident peritoneal Mφ infected with large numbers of *M. leprae* in vitro for several days also become unresponsive to IFN-γ (13). The mechanism of this down-regulatory effect by *M. leprae* is not clear; however, subversion of IFN-γ responsiveness is a survival technique that has been used by other intracellular pathogens (33–35). The inability of IFN-γ ACT human Mφ to kill *M. tuberculosis* has been attributed to this pathogen’s ability to disrupt IFN-γ signal transduction (35), impair CD64 transcription and surface expression (36), and induce IL-6 production (37). *M. leprae*-infected Mφ produce copious amounts of PGE_2_, the presence of which correlates both with Mφ in vitro unresponsiveness to IFN-γ activation (12) and successful T cell adoptive transfer into *M. leprae*-infected nu/nu mice (38).

The in vitro studies presented here support previous in vivo observations. Kinetic experiments in nu/nu mice following the traffic of labeled promonocytes into the infected footpad determined that 15–20% of the *M. leprae*-infected granuloma Mφ were <5 days old (39). Furthermore, IFN-γ treatment of these mice significantly enhanced the infiltration of Mφ into the footpad granuloma because 25–35% of the *M. leprae*-burdened Mφ were now newly arrived cells. Thus, the footpad granuloma is a highly dynamic entity containing numerous cells with continuous turnover and replacement of infected Mφ with fresh Mφ, and this dynamic nature can be manipulated experimentally with cytokine treatment. Because the *M. leprae*-engorged Mφ cannot become ACT by IFN-γ, these data imply that any killing, breakdown, or clearance of *M. leprae* from highly bacilliferous tissues resulting from immunotherapeutic or chemotherapeutic intervention would likely be accomplished by the newly arrived, competent Mφ ACT before or shortly after their traffic into the lesion.

Cytokine immunotherapy in leprosy patients further substantiates a dynamic nature for the *M. leprae*-induced granuloma. Enhancement of CMI and upgrading of clinical and histopathological classification has been attempted in borderline lepromatous and LL patients via cytokine immunotherapy. Upon injection of IFN-γ or IL-2 into leprosy lesions, a marked cellular infiltration occurred (23, 24, 40), and the bacillary index, a measure of *M. leprae* burden, was reported to markedly decrease (41). Thus, treatment of lesions with Th1 cytokines apparently evoked a CMI response, but the effect was transient; the specific unresponsiveness to *M. leprae* Ags characteristic of multibacillary disease was not reversed. It was proposed that the cytokine treatments, the IL-2 treatments in particular, may have induced a population of lymphokine-activated killer (LAK) cells that were responsible for the destruction of the *M. leprae*-infected Mφ (42). Studies in experimental leprosy have also demonstrated that NK and LAK cells can lyse *M. leprae*-infected Mφ (43, 44). However, it is unlikely that granuloma-derived NK cells played a role in our in vitro system. Flow cytometric analyses revealed that NK cells constituted only a very
minor population of our granuloma cell preparations (NK: Mφ, <0.001:1), a ratio well below that needed for effective NK or LAK cell lysis of mycobacteria-infected target cells (44–46). Furthermore, no IL-2, the cytokine that promotes differentiation of NK cell lysis of mycobacteria-infected target cells (44–46), a ratio well below that needed for effective NK or LAK cell lysis of mycobacteria-infected target cells (44–46).

We have previously shown that the ability of the ACT Mφ to inhibit M. leprae metabolism is highly dependent on the generation of RNI (29, 32). The current studies demonstrate that the inhibitory effects exerted by the ACT effector Mφ were also dependent on the production of RNI. In contrast, killing of the target cell-derived bacilli did not require a phagocyte oxidase-dependent respiratory burst. Peroxinitrite, formed by the interaction of NO and superoxide (47), is a potential product whereby ACT Mφ may exert their antimicrobial effects. However, the inability to generate superoxide by either the ACT phox91−/− effector Mφ (48, 49) or the M. leprae-infected footpad granuloma target Mφ (11, 12) would argue against peroxinitrite as the active toxic molecule in our system.

Although NO is a freely diffusible product of ACT Mφ, its reactivity depends largely on its concentration and the close proximity of the target cells (50). Our studies show that, in addition to RNI, intimate cellular contact between the M. leprae-infected target Mφ and the ACT effector Mφ was required. If the target and effector Mφ were physically separated using Transwell inserts, M. leprae retained high metabolic activity even in the presence of ACT Mφ generating high levels of RNI. Interestingly, cell-to-cell contact was also requisite for the enhancement of M. leprae metabolism by normal effector Mφ.

The importance of TNF-α in cellular recruitment to the site of infection, and subsequent granuloma formation and maintenance in response to mycobacterial infection has been demonstrated in numerous investigations (51–55), and TNF-α is expressed across the leprosy spectrum, both in human (56) and experimental (Ref. 27; L. B. Adams, N. A. Ray, D. M. Scollard, and J. L. Krahenbuhl, manuscript in preparation) leprosy. In the present study, M. leprae-infected granuloma Mφ synthesized large amounts of TNF-α upon ex vivo culture. TNF-α levels were reduced, however, upon coculture with effector Mφ. A likely explanation is that the TNF-α generated by the target Mφ was bound by receptors expressed by both normal and ACT effector Mφ. Interestingly, addition of anti-TNF-α Ab, although blocking TNF-α reactivity, did not inhibit the enhancing or detrimental effects of normal or ACT effector Mφ, respectively, on target Mφ-derived M. leprae, nor did it affect the levels of nitrite generated.

Although there were no T cells in our system, production of IFN-γ was evaluated, because several groups have reported that murine Mφ can produce IFN-γ under certain conditions. Treatment of Mφ with LPS (57) or IL-12 and IL-18 (58) induces both IFN-γ-specific mRNA expression and protein production. Stimulation with IFN-γ itself also resulted in the generation of IFN-γ by Mφ, presumably by an autocrine mechanism (59). Furthermore, Wang et al. (60) demonstrated an IL-12-dependent production of IFN-γ by alveolar Mφ from Mycobacterium bovis bacillus Calmette-Guérin-infected mice. In our system, M. leprae-infected granuloma Mφ did not themselves generate IFN-γ in vitro. However, low levels of IFN-γ were found in the cocultures, especially in the cocultures of target granuloma Mφ with ACT effector Mφ. Even though granuloma footpad target Mφ are refractory to exogenously added IFN-γ, the possibility that they may become ACT cells under our coculture conditions was addressed. This IFN-γ did not contribute to the inhibition of M. leprae metabolic activity, because addition of anti-IFN-γ Ab, although blocking IFN-γ reactivity, did not reverse killing of target cell-derived bacilli.

Especially noteworthy is the acquisition of M. leprae by the normal effector Mφ and the subsequent augmentation of M. leprae metabolic activity. M. leprae multiplies essentially unchecked in LL, and it has been postulated that Mφ turnover in the lepromatous
lesions results from uptake of bacilli that have been released from infected Mϕ that have lysed due to the overwhelming bacterial load in the cells. However, results presented in this study suggest that normal effector Mϕ may play a more active role in Mϕ turnover. *M. leprae* have an extremely slow growth rate in vivo and do not multiply in vitro (61). This was confirmed by the relatively constant metabolic rate of *M. leprae* harvested over the 5-day cocultivation period from our target cell-only cultures. Furthermore, this constant metabolic activity indicates that there was no spontaneous lysis of the *M. leprae*-infected target Mϕ, which would have resulted in a decrease in recovered bacilli and a drop in metabolic activity over time. Interestingly, even ACT Mϕ, in the presence of RN1 inhibitors or if NOS2−/− derived, could augment *M. leprae* viability. Whether or not a succession of challenges with normal Mϕ could sustain *M. leprae* viability in vitro for a prolonged period of time is an intriguing possibility.

The current understanding of the role of CMI in paucibacillary leprosy is drawn from both human and animal studies and suggests that cytotoxic CD4+ and/or CD8+ T lymphocytes or NK and LAK cells destroy the infected and incapacitated host cell (e.g., Mϕ or Schwann cell) and release the intracellular bacilli (14–16, 44). However, in the aftermath of the lysis of the *M. leprae*-infected cell, there is little direct evidence for the fate of the released bacilli or for a continued role for the Mϕ. Pathogens, including mycobacteria, may be killed directly by products of CTLs. Granulysin, a protein in the granules of T cells and NK cells from humans (there is no homolog for granulysin in mice), is lytic against a variety of tumor cells (62) and has been shown to directly kill extracellular *M. tuberculosis* and, after delivery by perforin, intracellular *M. tuberculosis* as well (63, 64). Evidence has been presented for granulysin-expressing T cells in skin lesions of patients with TT disease (65), although no direct evidence for killing of *M. leprae* was presented. But we emphasize that the present report is concerned with a model for polar LL, where *M. leprae*-specific T cells are lacking entirely and target Mϕ contain hundreds of bacilli. Although cytotoxic T cell participation is not explored in the current study, our findings support an important Mϕ-mediated event in the microenvironment of the granuloma. Upon destruction of the infected Mϕ by CTLs, the bacilli are released into the extracellular space where they can be rephagocytized by new, normal effector Mϕ. Munn and Cheung (67) have demonstrated both Ab-dependent and Ab-independent phagocytosis of *M. leprae* by Mϕ. A third mechanism of action could be the induction of apoptosis or necrosis in the target Mϕ and the phagocytosis of the infected apoptotic/necrotic cells (68, 69). Perskvist et al. (70) have demonstrated the uptake of *M. tuberculosis*-infected apoptotic neutrophils by Mϕ, and Fratazzi et al. (71) reported the adherence of uninfected Mϕ to *Mycobacterium avium*-infected apoptotic Mϕ and the subsequent inhibition of mycobacterial growth. Goldmann et al. (72) have shown that dendritic cells phagocytize bacillus Calmette-Guérin-infected necrotic Mϕ and present Ag to specific T cells. These possibilities are currently under investigation.

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