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Cytokine Activation Leads to Acidification and Increases Maturation of \textit{Mycobacterium avium}-Containing Phagosomes in Murine Macrophages\textsuperscript{1}

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\textit{Mycobacterium avium} (MAC) organisms multiply in phagosomes that have restricted fusigenicity with lysosomes, do not acidify due to a paucity of vacuolar proton-ATPases, yet remain accessible to recycling endosomes. During the course of mycobacterial infections, IFN-\(\gamma\)-mediated activation of host and bystander macrophages is a key mechanism in the regulation of bacterial growth. Here we demonstrate that in keeping with earlier studies, cytokine activation of host macrophages leads to a decrease in MAC viability, demonstrable by bacterial esterase staining with fluorescein diacetate as well as colony-forming unit counts from infected cells. Analysis of the pH of MAC phagosomes demonstrated that the vacuoles in activated macrophages equilibrate to pH 5.2, in contrast to pH 6.3 in resting phagocytes. Biochemical analysis of MAC phagosomes from both resting and activated macrophages confirmed that the lower intraphagosomal pH correlated with an increased accumulation of proton-ATPases. Furthermore, the lower pH is reflected in the transition of MAC phagosomes to a point no longer accessible to transferrin, a marker of the recycling endosomal system. These alterations parallel the coalescence of bacterial vacuoles from individual bacilli in single vacuoles to communal vacuoles with multiple bacilli. These data demonstrate that bacteriostatic and bactericidal activities of activated macrophages are concomitant with alterations in the physiology of the mycobacterial phagosome. The \textit{Journal of Immunology}, 1998, 160: 1290–1296.

\begin{itemize}
\item Follwing phagocytosis, \textit{Mycobacterium} species reside and multiply in phagosomes of the host’s macrophages (1). Mycobacteria-containing phagosomes have unique characteristics that support the intracellular survival and growth of these pathogens in professional phagocytes. Previous studies established that phagosomes containing MAC\textsuperscript{4} and \textit{Mycobacterium tuberculosis} have restricted fusigenicity with lysosomes (1–6) and do not acidify (2, 7, 8) due to a block in accumulation of vacuolar proton-ATPase (2). Despite the apparent sequestration of MAC and \textit{M. tuberculosis} vacuoles outside the endosomal/lysosomal continuum, recent studies have shown that these vacuoles are relatively dynamic, maintaining access to glycosphingolipids and glycoconjugates (9) from the host cell plasmalemma.

Immunoelectron microscopical studies by Clemens and Horwitz (4) on \textit{M. tuberculosis}-infected MO revealed the presence of MHC class II and transferrin receptor in mycobacteria-containing phagosomes. Indeed, we demonstrated recently that MAC phagosomes, although restricted in acquisition of proton-ATPases, have access to cathepsins B, L, and D which enter phagosomes early in their maturation (10). In spite of this, the high phagosomal pH limits processing and activation of cathepsin D. The hypothesis that mycobacterial phagosomes represent early endosomes stabilized in this stage was given further credence in two studies showing their accessibility to transferrin, a marker for the recycling endosomal system (10, 11).

Several independent studies published during the past 10 years have highlighted the role of certain cytokines in mycobacterial infections. It is accepted that activation of MO by T cell-, NK cell-, or macrophage-derived cytokines such as IFN-\(\gamma\), IL-1, granulocyte-macrophage-CSF, and TNF-\(\alpha\), alone or in concert, can contribute to the antimycobacterial potential of these cells, resulting in control of the infection in vitro (12–19) and in vivo (20, 21). Here, we have studied the influence of MO activation by IFN-\(\gamma\) and LPS on the maturation of MAC phagosomes and correlated these changes with mycobacterial survival. The data presented suggest that MAC phagosomes are shifted from an early to a late endosomal stage of phagosome maturation by MO activation, which is concomitant with a reduction in mycobacterial growth and viability.

\item Materials and Methods

Materials

The following Abs were used in this study: the mAb ID4B against LAMP-1 was obtained from the Developmental Hybridoma Bank, Iowa City, IA; mouse mAbs E11 and H9 against the vacuolar proton-ATPase E subunits were generous gifts from Dr. S. Gluck (Washington University, St. Louis, MO); rat mAb against transferrin receptor (R17/18, Tib217) were obtained from American Type Culture Collection, mouse mAb anti-digoxigenin was purchased from Boehringer Mannheim, Indianapolis, IN. The rabbit polyclonal Ab to cathepsin D was a generous gift from Dr. S. Kornfeld (Washington University). Secondary species-specific Abs labeled

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\item Abbreviations used in this paper: MAC, \textit{Mycobacterium avium} complex; LAMP 1, lysosome-associated membrane protein 1; NHS, N-hydroxysuccinimide; MO, bone marrow-derived macrophages; \(\alpha\)-m, \(\alpha\)-macroglobulin; NO, nitric oxide; iNOS, inducible nitric oxide synthase.

\end{itemize}
with horseradish peroxidase were purchased from Jackson Immunoresearch Laboratories, West Grove, PA. Iron-loaded transferrin and human α-in were both purchased from Calbiochem, San Diego, CA.

**Bone marrow-derived MO and activation**

MO were differentiated and maintained in culture as described previously (3, 9). MO were grown in bacteriologic petri dishes and split by placing them in cold PBS for 30 min followed by gentle scraping. After splitting, MO were allowed to establish a monolayer for at least 3 days in cell culture flasks before use. MO were activated according to the following procedures: For early time points (2–4 h), macrophages were incubated for 16 h with 400 U/ml recombinant mouse IFN-γ and 200 to 500 ng/ml LPS for 2 h before infection. For late time points (5 d), macrophages were incubated with 400 U/ml recombinant mouse IFN-γ and 200 to 500 ng/ml LPS added on day 4 postinfection.

**Bacteria, infection, colony-forming units**

MAC 101 from frozen stocks derived from the first passage following isolation from a mouse was cultured in Middlebrook broth (Difco, Detroit, MI) and used within 3 days of thawing. Translucent colony appearance as an indication of virulence was tested before each experiment and routinely revealed <1% opaque (avirulent) colonies. MO cultures were infected with MAC 101 in a 10:1 ratio in DMEM without antibiotics supplemented with 5% L929 conditioned medium, 5% horse serum for 2 h, washed twice, and cultured for the time period indicated for each experiment.

MO cultures (1 × 10^6) were set up in 12-well tissue culture plates in duplicate and infected with MAC in a bacteria-MO ratio of 10:1. CFUs were determined as follows. The infected MO monolayers were lysed in 1 ml of PBS containing 0.5% Nonidet P-40, and lysates were passaged seven times through a 25-gauge tuberculin needle. The lysates were diluted in Middlebrook broth (1/100; 1/1000; 1/10,000), and 100–μl aliquots were plated in duplicate onto Middlebrook agar plates and incubated for 10 days.

**Viability stain for mycobacteria**

Following the protocol of McDonough and Kress (22), infected MO were incubated in DMEM/10% FCS containing 4 mg/ml carboxyfluorescein diacetate (Molecular Probes, Eugene, OR) for 10 min at 37°C, fixed in 4% formaldehyde, and counterstained with Evans blue (Sigma Chemical Co., St. Louis, MO). Mycobacteria-infected MO were examined under an epifluorescence microscope and scored blind for strong (metabolically active) or low/no fluorescence (metabolically inactive).

**pH measurement**

MAC labeled with NHS-carboxyfluorescein (Boehringer Mannheim) were used to infect MO on quartz glass. The fluorescence of the total cell population was measured at different time points fluorometrically and compared with a standard pH curve using NHS-carboxyfluorescein-labeled MAC in suspension and in nigericin-treated MO, as described previously (2, 23).

**Protein labeling**

Transferrin was labeled covalently with NHS-digoxigenin (Molecular Probes) at a 10:1 molar excess in PBS, pH 7.8, for 30 min on ice and purified over Kwiksep exocellulose columns (Pierce Chemical Co., Rockford, IL). Human α-in was labeled with 125I (Amersham, Arlington Heights, IL) by iodobeads (Pierce Chemical Co.) according to the manufacturer’s instructions.

**Phagosome isolation**

Phagosomes containing MAC were isolated according to a protocol described earlier (9, 10). Contamination of phagosomes with other cellular material or digoxigenin-transferrin was evaluated for each experimental preparation by a crossover method described (9, 10). In brief, bacilli were added to unlabeled macrophages that were scraped and combined with an equal number of macrophages that were either labeled metabolically with [35S]methionine or incubated in digoxigenin transferrin. The level of contamination was 3 to 10% for metabolically labeled macrophages and below the level of detection for digoxigenin-transferrin macrophages.

**PAGE and Western blot**

Isolated phagosomes were lysed in 3× SDS buffer, boiled, and separated by SDS-PAGE (12%) under reducing conditions. After blotting onto nitrocellulose, blots were blocked in PBS containing 0.05% Triton X-114, 0.05% Tween-20, 10% goat serum, and 5% milk powder; incubated in the respective Abs; and developed using the Lumiglo system (Pierce Chemical Co.).

**Electron microscopy**

Macrophages activated before or postinfection were fixed in 2% glutaraldehyde in PBS, osmicated, dehydrated through ethanol, and embedded in Spurr’s resin. Thin sections were cut, contrasted with uranyl acetate and Reynolds’s lead, and examined in a Jeol 100CX electron microscope. The distribution of bacteria/vacuoles were scored by examining 200 vacuoles for each condition. No more than 6 vacuoles were scored per cell.

**Results**

**Effects of MO activation on MAC viability**

To study the effect of MO activation on MAC infection in vitro, we performed infection experiments on both resting and activated murine MO. Preliminary experiments confirmed previous reports that activation with IFN-γ (400 U/ml) alone postinfection did not induce a strong microbicidal response or the marked alteration in vacuole physiology detailed below. Full mycobacterial activity and iNOS expression requires a second signal, TNF-α, which can be induced by LPS (24, 25). We therefore added LPS at 200 ng/ml in concert with IFN-γ to maximize MO stimulation. Cell monolayers of MO were infected in a ratio of 10 bacilli/MO which infected >90% of the cells. MO were cultivated in medium plus or minus IFN-γ, and LPS was added before infection or 4 days postinfection (Fig. 1). In resting MO cultures, the number of recoverable CFU showed a modest decline over the first 4 days until the numbers could be seen to be increasing from 5 days postinfection. In contrast, in activated MO, this decline was more pronounced and sustained until the macrophage monolayer started to disintegrate at 6 days postinfection. Despite the ability of activated macrophages to regulate the bacterial population, bacterial death was neither rapid nor efficient, and CFU analysis indicated that many bacteria persisted within activated macrophages.

The initial stages of infection were characterized by static bacterial numbers, even in resting macrophages. This bacterial population is likely to be extremely heterogeneous with respect to viability. To reduce the heterogeneity of the bacterial population under study, we commenced analysis with addition of IFN-γ and LPS 4 days postinfection when the bacteria were entering exponential growth phase (Fig. 1). In nonactivated MOs, CFUs increased from 4 days postinfection and stayed at high levels until 10 days postinfection. In contrast, the CFUs from MO cultures activated 4 days postinfection peaked 2 days after activation and slowly decreased until 6 days after activation (10 days postinfection) (Fig. 1). During this same time, bacteria in resting MO had entered into exponential growth. To further characterize the effect of MO activation on MAC viability, we used a viability stain method based on bacterial esterase activity (22). The proportion of metabolically active (esterase-positive) mycobacteria increased during the entire observation period in resting MO; whereas in MO activated 4 days postinfection, the number of metabolically active mycobacteria stayed low and decreased until 6 days postactivation (10 days postinfection) (Fig. 1b).

Although the trend is comparable, the viability stain method “overestimated” the relative number of live bacilli in activated macrophages relative to the CFU data. This discrepancy may reflect bacterial aggregation on isolation from activated macrophages before plating or persistence of esterase activity in non-replicative bacteria. Despite this variation, both data sets argue that activation with IFN-γ and LPS renders murine bone marrow-derived MO capable of controlling an established MAC infection.
MAC are unable to block phagosome acidification in activated macrophages

In resting macrophages, phagosomes containing inert particles, or other pathogens like *Leishmania*, rapidly acidify from the extracellular pH to below pH 5.0 (2). In contrast, the pH within phagosomes containing MAC shows a more restricted drop and equilibrates to pH 6.2 (2). In this study, we compared the intraphagosomal pH of MAC phagosomes in resting vs activated MO. Resting MO were compared with MO activated for 16 h with IFN-γ and for 2 h with LPS before infection. Carboxyfluorescein-labeled MAC were bound to the MO on ice, unbound bacteria were washed off, and the MOs were placed at 37°C for the time periods stipulated. As detailed previously (2), the pH of MAC phagosomes in resting MO did not drop below pH 6.2 (Fig. 2). In contrast, in activated MO, the pH in the MAC-containing phagosomes dropped to pH 5.2 within 180 min of internalization (Fig. 2). There was an intriguing “rebound” in the pH at 90 to 120 min postinfection which was observed in three independent experiments.

Acidification of MAC phagosomes correlates with acquisition of vacuolar proton-ATPases

We had attributed the restricted acidification observed in mycobacteria phagosomes to the paucity of vacuolar proton-ATPases in this compartment (2). To test whether MO activation reverses this phenotype, similar numbers of phagosomes were isolated from resting MO or from MO activated with IFN-γ for 16 h and LPS for 2 h before infection. Carboxyfluorescein-labeled MAC were bound to the MO on ice, unbound bacteria were washed off, and the MOs were placed at 37°C for the time periods stipulated. As detailed previously (2), the pH of MAC phagosomes in resting MO did not drop below pH 6.2 (Fig. 2). In contrast, in activated MO, the pH in the MAC-containing phagosomes dropped to pH 5.2 within 180 min of internalization (Fig. 2). There was an intriguing “rebound” in the pH at 90 to 120 min postinfection which was observed in three independent experiments.

Data represent the mean of measurements from three independent experiments for each time point.
MO undergo a functional translocation to a later endosomal stage, this should be mirrored by a loss of accessibility to transferrin. To test this, both resting and activated MO infected with MAC 5 days previously were incubated with digoxigenin-labeled transferrin for 45 min, washed extensively, and lysed. Comparable numbers of phagosomes from resting vs activated MO were separated by SDS-PAGE, blotted, and probed for digoxigenin. MO lysates revealed that similar amounts of digoxigenin-transferrin were taken up by both populations of MO (Fig. 4c). Also, as described previously, transferrin was readily detected in MAC phagosomes from resting MO. In contrast, only minimal amounts of digoxigenin-transferrin were present in MAC phagosomes from activated MO (Fig. 4c). These were the same phagosome preparations probed for proton-ATPase (E subunit) and LAMP 1 as discussed. These data suggest that in activated MO, MAC-containing phagosomes are shifted functionally toward a late stage in endosomal maturation characterized by accumulation of proton-ATPases and the loss of interaction with transferrin-carrying vesicles. Additional experiments were performed using 125I-labeled α_m, which usually proceeds along the lysosomal pathway following endosomal uptake. As expected, only small amounts of α_m could be detected in isolated MAC phagosomes from resting MO, whereas up to four times more α_m was found in phagosomes from activated MO (data not shown).

**Alterations in phagosome physiology appears to precede the drop in MAC viability**

It is still unclear whether the increased maturation of MAC phagosomes is symptomatic of or causal to the loss of bacterial viability. The complex responses of macrophages to cytokine activation render this question difficult to resolve. Furthermore, there are no data regarding the effects of macrophage activation on the regulation of phagosome/lysosome fusion independent of mycobacterial infections.

However, electron microscopic analysis of infected MO shortly after activation indicates that one of the first phenotypic alterations in activated macrophages is the coalescence of individual *M. avium*-containing vacuoles into communal vacuoles with many bacilli (Figs. 5 and 6). This is observed in macrophages activated before infection and in macrophages activated 4 days postinfection and examined 5 days postinfection. Quantitation of the distribution of bacilli in vacuoles (Fig. 6) demonstrates that at early time points, 2 h postinfection, there is a marked decrease in the numbers of bacilli in individual vacuoles in activated vs resting MO. Furthermore, the more established the infection, such as 5-day infections in resting MO, the higher is the proportion of single bacteria in individual vacuoles. However, at both the 2-h and 5-day time points, the majority of bacilli show few signs of damage or degradation, even in the communal vacuoles of activated MO. These data provide a preliminary indication that the merging of vacuoles precedes any marked drop in bacterial viability, as assessed by CFUs shown in Figure 1.

**Discussion**

This study details experiments designed to examine the effects of MO activation on the biology of MAC-containing vacuoles and describes data that correlate the alterations in vacuole physiology with the subsequent development of mycobacteriostatic and mycobactericidal properties of the infected host MO.

The role of cytokine-activated MO in the modulation of mycobacterial infections shows qualitative variation with mouse strain and bacterial species but has been shown to be critical in protection through experiments conducted on IFN-γ and TNF-α receptor
knockout mice (26, 27). In vitro, IFN-γ- and/or TNF-α-activated murine MO are able to inhibit growth of *Mycobacterium bovis* and *M. tuberculosis* (12, 15–17, 26). Similarly, Appelberg and Orme (18) showed bacteriostasis by IFN-γ-activated murine MO toward some but not all MAC isolates tested. The levels of MO-derived TNF-α produced by infected MO varied with bacterial isolate and appeared to be crucially involved in the protective response (18, 19, 27). In systems where mycobactericidal behavior has been induced, there is some debate as to the mechanism(s) involved. Growth inhibition of *M. tuberculosis* and *M. bovis* by activated MO has been attributed to the release of NO but not to reactivated oxygen metabolites (16). In contrast, growth inhibition of MAC by

**FIGURE 5.** Electron micrographs of murine bone marrow-derived MO infected with *M. avium*, revealing alterations in vacuole morphology following activation of MO with IFN-γ and LPS. *a,* Resting MO 2 h following infection. The bacilli tend to be sequestered in individual vacuoles that show little evidence of lysosomal fusion. *b,* Activated MO 2 h following infection. The bacteria are observed more frequently in communal vacuoles that contain dense, lysosomal matrix. MO were activated with IFN-γ (400 U/ml) for 16 h and LPS (500 ng/ml) for 2 h before infection. *c,* Resting MO 5 days postinfection. *M. avium* persist and divide in individual vacuoles. Many of these replicating organisms have prominent ribosomes (arrowed). *d,* Activated MO 5 days postinfection. Again, there is a marked tendency for the bacteria to be in vacuoles containing multiple bacilli. Although there is little obvious degeneration of the bacilli, the ribosomes are not as numerous or developed as those seen in *c.* MO were activated on day 4 with IFN-γ (400 U/ml) for 16 h and LPS (500 ng/ml) for 2 h before processing. Comparable results were observed in two independent experiments.
activated MO has been suggested to be independent of NO (18) and mediated by superoxide production (19), possibly in the context of enhanced phagosome/lysosome fusion (18).

Despite the body of data demonstrating the central role of iNOS in the regulation of murine mycobacterial infections and other intracellular pathogens, the evidence is all of the same type, either protection through the use of inhibitors such as nonhydrolyzable arginine analogues or the use of iNOS knockout mice (28–30). Although these experiments all indicate that iNOS is necessary for protection, they do not shed light on whether it is sufficient. Obtaining observations (3). Xu, S., A. Cooper, S. Sturgill-Koszycki, T. vanHeyningen, D. Chatterjee, I. Orme, P. Allen, and D. G. Russell. 1994. Intracellular trafficking in Mycobacterium avium-infected macrophages. J. Immunol. 153:2568.

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