Hypoxia Triggers the Expression of Human β-Defensin 2 and Antimicrobial Activity against *Mycobacterium tuberculosis* in Human Macrophages

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Low oxygen tension is a nonspecific response to inflammatory stimuli, including malignant tumors, atherosclerosis, autoimmune diseases, wounds, and infections (1). The restriction of local oxygen supply initiates a genetic program that governs local immune responses. Specifically, macrophages are attracted, immobilized (2), and reprogrammed to a proinflammatory state that is characterized by the upregulation of proangiogenic molecules, PGs, cytokines, chemokines, and inducible NO synthase (3, 4).

To investigate the effect of oxygen restriction on host–pathogen interaction, we chose tuberculosis as a model for two reasons. First, several lines of evidence indicate that oxygen restriction is beneficial for the clinical outcome of tuberculosis. In rabbits and guinea pigs kept in cages with continuously low oxygen supply, the infection was markedly ameliorated in comparison with the control animals maintained in open cages (5). Similarly, low oxygen levels protected mice from infection with virulent Mycobacterium tuberculosis (6). Along these lines, the prevalence of tuberculin skin test positivity in humans was reduced in mountain dwellers compared with control villages at sea level, indicating that innate immune responses cleared the pathogen more efficiently in a hypoxic environment (7). It has also been speculated that low oxygen tension contributes to the salubrious effect of high-altitude sanatoria and the partial clinical success of pulmonary collapse therapy practiced in the preantibiotic era (8).

Because the host appears to benefit from the restriction of oxygen, the bacterium consequently seeks for areas with high oxygen concentrations. Specifically, tubercle bacilli tend to persist and reactivate in the highly oxygenized apex of the human lung (9). More definitive evidence for this phenomenon comes from historical experiments in which guinea pigs were forced to maintain an upright position during the infection. These animals were much more likely to develop disease in the apical areas of the lungs than were control animals (10).

The second rationale for studying the interaction of macrophages and M. tuberculosis under oxygen limitation is the hypoxic microenvironment present at the site of infection. The granulomas in guinea pigs, rabbits, nonhuman primates, and humans are characterized by low levels of oxygen (11, 12). In contrast, granulomas in mice infected with virulent M. tuberculosis are not hypoxic (11, 13).

In this study, we hypothesized that the oxygen concentration is a critical determinant for the interaction of macrophages with M. tuberculosis. Our results demonstrate that hypoxia activates the vitamin D-dependent antimicrobial pathway, resulting in up-regulation of human β defensin 2 (hBD2) and growth inhibition of intracellular M. tuberculosis. We conclude that the local oxygen tension modulates the efficacy of local immune responses, specifically the activation of antimicrobial effector mechanisms.

Materials and Methods

Cell culture reagents

Cells were cultured in RPMI 1640 supplemented with glutamine (2 mM), Sigma), 10 mM HEPES, 13 mM NaHCO₃, 100 μg/ml streptomycin, 60 μg/ml penicillin (all from Biochrom) and 5% heat-inactivated human AB serum (Cambrex) (= complete medium [CM]). The 25 (OH) vitamin D concentration in the human serum was 41 nmol/l, and the 1,25(OH)₂ vitamin concentration was 0.13 nmol/l, as determined by RIA (Immunodiagnostic Systems). The same batch of human serum was used throughout the study. For experiments involving M. tuberculosis, the medium was modified to optimize phagocytosis (non-heat–inactivated serum) and allow multiplication of the bacteria (no streptomycin). 1α, 25-dihydroxyvitamin D3 [1,25(OH)₂ VitD] was purchased from Biomol.
**Hypoxia chamber**

A hypoxia chamber was tailor-made for the specific requirements of a biological safety level 3 facility (Toepffer Laboratories). The chamber represents a closed system that is accessible from the outside through aerosol tight gates. Outgoing air is filtered through high-efficiency particulate air filters, and samples or controls were performed in triplicates. Viability (%) was calculated according to the formula: OD samples − OD medium alone/OD Triton-treated wells − OD medium alone × 100. All samples and controls were performed in triplicates.

**Preparation and viability of macrophages**

PBMC were isolated by density-gradient centrifugation of buffy coat preparations from the blood of healthy donors. Monocytes were isolated by adherence on plastic and cultured in the presence of GM-CSF (10 ng/ml; Berlex) for 4–6 d. Monocyte-derived macrophages (MDM) were detached with EDTA (1 mM; Sigma) and cultured at 1–105 in 500 μl CM in 24-well plates at 20 or 1% oxygen tension. Viability of MDM after 6 d of culture was determined using the lactate dehydrogenase (LDH) cytotoxicity detection assay (Clontech), according to the instructions of the manufacturer. Briefly, supernatants (200 μl) of the MDM cultures were harvested, centrifuged, and transferred into 96-well plates. To determine the maximal LDH release, 1% Triton X-100 (Sigma) was added to selected wells. Substrate was added, and the samples were incubated for 30 min. Prior to measurement of OD in an ELISA reader (wavelength 492 nm) the maximal LDH release, 1% Triton X-100 (Sigma) was added to selected wells. The metabolic activity of extracellular M. tuberculosis was estimated by incubation of bacilli (2 × 109/well) in 7H9 broth (BD Biosciences) supplemented with 1% glyceral (Roth), 0.05% Tween 80 (Sigma), and 10% Middlebrook oleic acid, albumin, dextrose, and catalase enrichment (BD Biosciences). Aliquots from logistically growing cultures were frozen in PBS/10% glycerol, and representative vials were thawed and enumerated for viable CFU on Middlebrook 7H11 plates. For measuring the intracellular growth of M. tuberculosis, macrophages were infected with single-cell suspensions of M. tuberculosis (multiplicity of infection [MOI] 5). After 18 h, macrophages were washed three times to remove extracellular bacteria. The efficacy of infection was determined in each experiment by acid fast stain (auramine rhodamine; Merck) and was 31 ± 16%. To enumerate the number of viable bacilli after 4–6 d of incubation, the infected macrophages were lysed with 0.3% saponin (Sigma) to release intracellular bacteria. Cell lysates were resuspended vigorously, transferred into screw caps, and sonicated in a preheated (37°C) water bath for 10 min. Aliquots of the sonicate were serially diluted (1:10, 1:100, 1:1000) in CM without streptomycin. Four dilutions of each sample were plated in duplicates on 7H11 agar plates and incubated for 14 d. The concentration of TNF (ThermoScientific), IL-10 (DuoSet, R&D Systems), IL-12 (eBioscience), and hBD2 (PeproTech) was determined by sandwich ELISA, exactly as recommended by the manufacturer. Sensitivities were 16 pg/ml (TNF), 8 pg/ml (IL-10), 32 pg/ml (IL-12), and 12 pg/ml (hBD2).

**Quantitative LightCycler PCR**

MDM (3 × 105) were infected with M. tuberculosis (MOI 5) for 16 h in six-well plates, harvested, and distributed in 24-well plates at 7.5 × 105 cells/well. After 24 h of culture at 20 or 1% oxygen, MDM were lysed using TRIzol reagent, according to the manufacturer’s protocol. After DNAse treatment (New England Biolabs), cDNA was prepared by reverse transcription (Fermentas). LightCycler PCR was performed using SYBR Green PCR Master Mix (Roche). The following primers were used: forward β-actin, 5′-GGCCACGCGGTCTCT-3′; reverse β-actin, 5′-GGTGAGCGTACAGGTTTTC-3′; forward vitamin D receptor (VDR), 5′-A-AGGCGCAACGACCCACT-3′; reverse VDR, 5′-ATCATGCCGATGT-CCACACA-3′; forward hBD2, 5′-GGTTGTGTTTTGTTGTTGTTAAGCCG-3′; reverse hBD2, 5′-AGGACCGAGACTGGATGACA-3′; forward cathelicidin, 5′-GGACCGACCGACCCAAA-3′; and reverse cathelicidin, 5′-GACCTGCTCTCTCCACTGGA-3′. The cycles were performed as follows: 1 cycle 95°C, 10 min; 40 cycles: 95°C, 10 s; 60°C, 10 s; 72°C, 6 s. The mRNA levels for VDR, hBD2, and cathelicidin were normalized to the amount of β-actin, which was measured simultaneously. A comparative threshold cycle was used to determine gene expression relative to the uninfected MDM cultured at 20% oxygen. The relative expression levels were calculated as described previously (14).

**Statistical analysis**

The results are presented as mean ± SD. The Student t test was used to determine statistical significance between differentially treated cultures. Differences were considered significant at p < 0.05.

**Results**

**Survival of extracellular M. tuberculosis is not affected by the restriction of oxygen**

To assess whether low oxygen tension influences the growth of extracellular M. tuberculosis on agar plates, a defined number of bacilli (400 CFU) was inoculated on 7H11 plates and cultured for 14 d at 20 or 1% oxygen. At 20% oxygen, we observed the typical dry, colorless colonies of variable size, with diameters ranging from 1 to 3 mm (Fig. 1A). In contrast, colonies on plates cultured at 1% oxygen were much smaller (<1 mm), and a magnifying glass was required for precise quantification. Nevertheless, the number of colonies, reflecting the number of viable bacilli, was essentially identical in both groups (Fig. 1B). Accordingly, extended culture of plates at 1% oxygen for up to 4 wk yielded no CFU (mean ± SD) calculated from four experiments.
colonies of similar size as were observed after 2 wk of culture at 20% oxygen tension (data not shown). This observation suggested that metabolic activity, but not survival of the bacilli, was reduced by the restriction of oxygen supply.

Reduced metabolic activity, but sustained survival, of extracellular M. tuberculosis under hypoxia

To determine the metabolic activity of M. tuberculosis, we incubated mycobacterial cultures in liquid broth for 4 d at 20, 10, 5, 1, or 0.5% oxygen or in its absence. As an estimate for metabolic activity, we measured the incorporation of [3H]uracil into the mycobacterial RNA during the final 18 h of incubation (Fig. 2A). At 10% oxygen, the metabolic activity decreased sharply by 73 ± 10% compared with the control cultures at 20% oxygen and persisted at this level down to 0.5% oxygen. In the complete absence of oxygen (GasPak chamber), the metabolic activity was at background levels (heat-killed bacilli). In contrast, the number of cultivable bacilli was barely reduced after 4 d of culture at 10, 5, 1, or 0.5% oxygen compared with 20% oxygen (Fig. 2B). In agreement with the lack of metabolic activity, the mycobacteria did not survive anoxia for 4 d. We conclude that low levels of oxygen are sufficient for the survival, but not for the multiplication, of M. tuberculosis.

Macrophage viability is not affected by low oxygen tension

Because M. tuberculosis is an intracellular pathogen, we next focused on the interaction of the bacilli with its primary host cell, the macrophage. Based on our previous results and the predicted oxygen environment in vivo, we selected to compare the interaction between virulent M. tuberculosis and primary human macrophages at 20 and 1% oxygen tension. As expected, hypoxia inducible factor (HIF)-1α, a surrogate marker for hypoxia (15), was upregulated when MDM were cultured at 1% oxygen, demonstrating that our conditions trigger the macrophage hypoxia program (data not shown).

To determine whether the continuous restriction of oxygen interferes with the viability of human macrophages, we cultured MDM for up to 6 d at 1% oxygen and monitored the release of LDH as a marker for membrane leakage. Uninfected and M. tuberculosis-infected MDM remained as viable as did the controls cultured under standard conditions (Fig. 3). These results established that the continuous culture of MDM with virulent M. tuberculosis in a closed chamber adjusted to 1% oxygen tension is appropriate for studying immune responses under hypoxic conditions for up to 1 wk.

Phagocytosis of live M. tuberculosis is not influenced by oxygen tension

To investigate the functional impact of the hypoxia program on host–pathogen interaction, we next monitored key elements of the immune response: phagocytosis, cytokine release, and the expression of selected antimicrobial peptides.

MDM were cultured with live M. tuberculosis at increasing MOI (0.1, 1, and 10) in chamber slides and incubated overnight at 20 or 1% oxygen. Slides were stained with auramine rhodamine, and the number of infected MDM was counted using a confocal laser microscope (Fig. 4). The number of MDM that harbored M. tuberculosis increased from 1.9 ± 0.2% (MOI 0.1) to 33 ± 3% (MOI 1) to 92 ± 2% (MOI 10) when cultured at 1% oxygen, and it did not differ significantly from cells cultured at 20% oxygen. Similarly, the number of bacilli per infected MDM was similar at 20% oxygen (2.3 ± 1.4) and 1% oxygen (2.1 ± 1.6) (data not shown).

Cytokine release is not affected by low oxygen tension

The pattern of cytokines that is released early after phagocytosis of intracellular bacteria is critical for directing innate and adaptive immune responses. Therefore, we compared M. tuberculosis-triggered cytokine release at 20 and 1% oxygen tension. As surrogate cytokines for Th1- and Th2-dominated immune responses, we measured the concentration of TNF, IL-12 (Th1), and IL-10 (Th2) in the supernatants after overnight infection (MOI 5) (Fig. 5). The levels of the Th1 cytokines TNF (20%: 6843 ± 896 pg/ml; 1%: 7234 ± 1649 pg/ml) and IL-12 (20%: 3958 ± 581 pg/ml; 1%: 3004 ± 172 pg/ml) were not significantly different between MDM cultured at 20% or 1% oxygen tension. Similarly, the concentration of the Th2 cytokine IL-10 did not differ between the groups.

FIGURE 2. Reduced metabolic activity, but sustained survival, of extracellular M. tuberculosis under hypoxia. Bacteria (3 × 10⁷) were plated in triplicates in a 96-well round-bottom plate and cultured for 4 d in 7H9 Middlebrook medium at the indicated oxygen levels. Because the oxygen chamber only permits the evaluation of one condition at a time, results for each O₂ level was compared with the standard oxygen tension (20%) of the respective experiment. (A) [3H]uracil (3 μCi) was added for the final 18 h of incubation. M. tuberculosis was then killed by paraformaldehyde, and the incorporated radioactivity was measured in a beta counter. The graph shows the percentage reduction of cpm (mean ± SD) compared with incubation at 20% oxygen. The experiment was performed three times, each in triplicates. The asterisks indicate significant differences compared with bacilli cultured at 20% oxygen. (B) Bacteria were plated at 5-fold dilutions on 7H11 agar plates, and the number of CFU was determined after 2 wk of culture at 20% oxygen. The graph shows the reduction of CFU (mean ± SEM) determined from three independent experiments, all performed in triplicates. The asterisks indicate a significant difference as compared to bacilli cultured at 10% oxygen.

FIGURE 3. Macrophage viability is not affected by low oxygen tension. MDM were infected (MOI 1) and cultured for 6 d at 20 or 1% oxygen tension. The viability was determined by measuring LDH release in the supernatant. Percentage viability was compared with the background levels of medium cultured in the absence of cells. (A) Uninfected cells. (B) Infected cells. Percentage of MDM viability (mean ± SD) calculated from five independent experiments.
Regulation of VDR and antimicrobial peptides at low oxygen tension

We recently identified an antimicrobial pathway that is triggered by VDR and results in the cathelicidin and hBD2-mediated killing of *M. tuberculosis* (16–18). To evaluate whether this cascade is affected by the restriction of oxygen supply, we cultured *M. tuberculosis*-infected MDM at 20 or 1% oxygen overnight and measured the expression of VDR by quantitative LightCycler PCR. Based on 14 independent experiments, we found that the mRNA for VDR was upregulated 3.1 ± 0.3-fold by infection with *M. tuberculosis* (Fig. 6A) compared with uninfected MDM. Upregulation was significantly more pronounced (5.8 ± 1.7-fold) when infected MDM were exposed to 1% oxygen.

Because VDR regulates expression of the antimicrobial peptides cathelicidin and hBD2 (16, 19), we next examined the mRNA expression of these two antimicrobial peptides at 20 and 1% oxygen tension. Using MDM derived from fresh monocytes, after 4 d of culture we found a significant downregulation (63 ± 21%; *p* < 0.05) of cathelicidin mRNA in *M. tuberculosis*-infected MDM cultured at 1% oxygen tension compared with MDM cultured at 20% oxygen (Fig. 6B). In contrast, the mRNA for hBD2 was reproducibly upregulated by infection with *M. tuberculosis* at 20% oxygen (2.0 ± 0.3-fold) (20), and it was much more pronounced at 1% oxygen (5.3 ± 1.8-fold; *p* < 0.001) (Fig. 6C, *n* = 14).

Because vitamin D levels in human serum were shown to be critical for the induction of cathelicidin and hBD2 (16, 18), we tested whether the addition of defined amounts of 1,25(OH)2 VitD, the biologically active form of vitamin D, had an effect on hBD2 and cathelicidin expression in *M. tuberculosis*-infected MDM. Even high concentrations of up to 10−7 M 1,25(OH)2 VitD did not influence the expression of hBD2 mRNA at 20 or 1% oxygen tension (Fig. 7A), reflecting the lack of the second signal, which is required for the upregulation of hBD2 in human macrophages (18). In contrast and as published previously (16, 18) 1,25(OH)2 VitD triggered cathelicidin expression in a dose-dependent manner at 20% oxygen (Fig. 7B). Upregulation of cathelicidin was still significant, but considerably less pronounced, under oxygen restriction (1%), extending our finding that hypoxia interferes with cathelicidin expression in *M. tuberculosis*-infected MDM (Fig. 6).

To determine whether increased mRNA expression of hBD2 translated into increased protein release, we measured the protein concentration in the supernatants after 48 h of culture. *M. tuberculosis* infection alone moderately increased hBD2 release at 20% O2 from <12 to 22 ± 5 pg/ml (Fig. 8). The amount of hBD2 increased to 60 ± 7 pg/ml when infected cells were incubated at 1% O2, demonstrating that increased mRNA expression of hBD2 resulted in increased transcription and release of the protein. Taken together, our functional studies demonstrated that viability, phagocytosis, and cytokine production of MDM tolerate remarkably low levels of oxygen. However, oxygen restriction induced the upregulation of VDR and modulated the expression of the antimicrobial peptides cathelicidin and hBD2, suggesting the intriguing possibility that the availability of oxygen affects the growth of intracellular *M. tuberculosis*.
Numerous studies reported that low oxygen levels shift macrophages toward an inflammatory state characterized by the release of proinflammatory cytokines (24–26), the upregulation of pattern-recognition receptors (27) and costimulatory molecules (28), and induction of genes involved in macrophage survival, tissue revascularization, and radical formation (1). In this study, we focused on functional experiments on the interaction of human macrophages with the virulent pathogen \textit{M. tuberculosis}. We show

\textbf{FIGURE 7.} Increased hBD2 release at 1% oxygen tension. MDM were infected with \textit{M. tuberculosis} (MOI 5) in bulk culture, harvested, and distributed at $2 \times 10^6$ in 500 µl CM/well. After 48 h of culture at 1% O$_2$, supernatants were harvested, sterile-filtered, and assayed for hBD2 concentration by ELISA (sensitivity 12 pg/ml). Control cultures were left uninfected and cultured at normoxia. The figure shows the mean ± SD calculated from the results of five donors.

\textbf{FIGURE 8.} 1,25(OH)$_2$ VitD induces upregulation of cathelicidin but not hBD2. MDM were infected with \textit{M. tuberculosis} (MOI 1), stimulated with indicated concentrations of 1,25(OH)$_2$ VitD, and cultured overnight at 20 or 1% oxygen tension. mRNA was prepared and analyzed for the expression of hBD2 mRNA (A) and cathelicidin mRNA (B). The figure shows the fold change compared with the mRNA levels in cells cultured in the absence of 1,25(OH)$_2$ VitD at the respective oxygen tension. The figures show the mean ± SD of five donors.

\textbf{FIGURE 9.} Decreased intracellular growth of \textit{M. tuberculosis} at 1% oxygen tension. MDM were infected in bulk culture overnight (MOI 1), harvested, and replated in 24-well plates ($1 \times 10^3/300 \mu$l). Infected cells were cultured for $5 \pm 1$ d at 20 or 1% oxygen, and the number of viable bacilli was determined by plating cell lysates on 7H11 agar plates. The number of CFU was determined after 2 wk of culture at 20% oxygen. (A) Individual results of 12 donors. (B) Summary of the results of all donors (mean ± SD).
that the initial response of macrophages to M. tuberculosis—uptake of the bacilli and cytokine release—is not influenced even at harsh oxygen conditions (1%). However, the restriction of bacterial growth, the key function that defines the quality of an effective immune response, was significantly enhanced. We established a hypoxia chamber that creates a constant and tightly controlled gaseous environment and at the same time fulfills the safety criteria required for working with virulent M. tuberculosis. Using this refined setting, we extend an earlier finding on the impaired growth of M. tuberculosis in human macrophages at 5% oxygen tension (29). It is possible that the reduced mycobacterial metabolism in a hypoxic environment contributes to this effect (23, 29) (Fig. 2). However the oxygen tension in the phagolysosome, the mycobacterial niche, is unlikely to reflect the atmospheric milieu, because it is influenced by complex intracellular metabolic processes triggered by host and pathogen. In addition, pathogens, such as M. tuberculosis, divert macrophage functions to secure their intracellular survival, multiplication, and spread (30), and the rate of extracellular multiplication will not necessarily mirror the fate inside the host cell.

We provide evidence that the activation of an innate immune effector pathway is also involved in the containment of the bacilli. Specifically, hypoxia triggered the upregulation of VDR and one of its downstream targets, the antimicrobial peptide hBD2 (Fig. 6). Because hBD2 has been associated with immune responses against M. tuberculosis (16, 20, 31), and hBD2-transfected macrophages restrict the growth of intracellular M. tuberculosis (32), our results suggest a mechanism by which oxygen limitation triggers hBD2, resulting in reduced multiplication of tubercle bacilli. In contrast, cathelicidin, which was shown to mediate VDR-dependent killing of M. tuberculosis (17), was downregulated under oxygen-limiting conditions. Intriguingly, this indicates a differential regulation of vitamin D-dependent antimicrobial peptides, possibly shaped by the surrounding cytokine milieu (33). Because both antimicrobial peptides are active against M. tuberculosis (16, 18), it was remarkable that antimicrobial activity was almost completely abrogated by silencing of cathelicidin in a human monocytic cell line (17). Silencing of both cathelicidin and hBD2 significantly reduced antimicrobial activity in TLR2/1-stimulated human monocytes compared with monocytes transfected with control small interfering RNA (18). Given the direct antimycobacterial activity of hBD2 and the enhanced mycobacterial growth in hBD2-deficient macrophages (18), we propose that the upregulation of hBD2 by hypoxia is one mechanism by which mycobacterial growth is reduced at hypoxia. However, our findings do not provide experimental proof for a direct link between increased hBD2 expression and reduced mycobacterial growth. The upregulation of the vitamin D-dependent antimicrobial pathway provides only one of several potential mechanisms for reduced multiplication of the bacilli. In addition to the direct effects of hypoxia on M. tuberculosis, alternative mechanisms include effects of oxygen availability on the iron metabolism, the production of indolamin dioxygenase, the modification of radical production (NO, oxygen intermediates), or intracellular-trafficking events, such as phagosome maturation or autophagy, all of which have been implicated in the elimination of intracellular M. tuberculosis (30).

Similarly to M. tuberculosis, the multiplication of the parasite Leishmania amazonensis in a human macrophage-like cell line and in dendritic cells is reduced at 5% oxygen tension (34, 35), corroborating the impression that hypoxia favors the antimicrobial activity of macrophages. In contrast, one report demonstrates an opposite effect on epithelial cells (36). Specifically, cells isolated from fallopian tubes and cultured at low oxygen conditions were permissive for the growth of the intracellular bacterium Chlamydia trachomatis. This was shown to be related to an impaired response to IFN-γ, resulting in reduced phosphorylation of STAT-1 and activity of indolamin dioxygenase (36). These few available studies suggest that antimicrobial defenses in epithelial cells are hampered by the restriction of oxygen, whereas they are strengthened in macrophages, possibly reflecting the elementary role of macrophages in the elimination of intracellular pathogens at inflammatory sites.

Indirect evidence for a role of oxygen tension during bacterial infections in vivo comes from studies with HIF-1α−/− deficient mice. These mice, which fail to adapt to oxygen deprivation, are more susceptible to infections with streptococci (37, 38), staphylococci (39), and enteric Yersinia (40). In the case of group A streptococci, the increased susceptibility to infection was associated with the failure of HIF-1α−/− deficient mice to upregulate the antimicrobial peptide CRAMP, the murine homolog of cathelicidin (39). However, these mice cannot be used to directly assess the effect of low oxygen tension, because HIF-1α is also activated under normoxic conditions in an inflammatory microenvironment (41), including infections with human pathogens (42).

In summary, we demonstrate that low oxygen levels, which are characteristic for tuberculous granulomas in human tissue, trigger the expression of VDR and the antimicrobial peptide hBD2 and killing of intracellular M. tuberculosis. This provides novel evidence that local oxygen deprivation is beneficial for macrophage functions and may contribute to the successful containment of M. tuberculosis during latent tuberculosis.

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