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IFN-γ–Dependent Activation of Macrophages during Experimental Infections by *Mycobacterium ulcerans* Is Impaired by the Toxin Mycolactone

Egídio Torrado,*[,1,2] Alexandra G. Fraga,*[,1] Elsa Logarinho,* Teresa G. Martins,* Jenny A. Carmona,* José B. Gama,* Maria A. Carvalho,† Fernanda Proença,† Antonio G. Castro,*[,3] and Jorge Pedrosa*[,3]

Buruli ulcer, caused by *Mycobacterium ulcerans* infections, is a necrotizing skin disease whose pathogenesis is associated with the exotoxin mycolactone. Despite the relevance of this emergent disease, little is known on the immune response against the pathogen. Following the recent demonstration of an intramacrophage growth phase for *M. ulcerans*, we investigated the biological relevance of IFN-γ and the antimycobacterial mechanisms activated by this cytokine in *M. ulcerans*-infected macrophages. Three *M. ulcerans* strains were tested: 5114 (mutant mycolactone-negative, avirulent strain); 94–1327 (intermediate virulence); and 98–912 (high virulence). We show in this study that IFN-γ is expressed in mouse-infected tissues and that IFN-γ–deficient mice display increased susceptibility to infection with strains 5114 and, to a lesser extent, 94–1327, but not with the highly virulent strain. Accordingly, IFN-γ–activated cultured macrophages controlled the proliferation of the avirulent and the intermediate virulent strains. Addition of mycolactone purified from strain 98–912 to cultures of IFN-γ–activated macrophages infected with the mycolactone-negative strain led to a dose-dependent inhibition of the IFN-γ–induced protective mechanisms, involving phagosome maturation/acidification and increased NO production, therefore resulting in increased bacterial burdens. Our findings suggest that the protection mediated by IFN-γ in *M. ulcerans*-infected macrophages is impaired by the local buildup of mycolactone. ***The Journal of Immunology*, 2010, 184: 947–955.**

Buruli ulcer (BU), caused by infections with *Mycobacterium ulcerans*, is a necrotizing disease of the skin and soft tissues that can cause extensive scars and disabilities if not treated at early stages. Although BU is far more common in Africa, it is also found in South America, Asia, and Australia (1). In endemic areas of Africa, BU can affect up to 22% of the population, being more prevalent than tuberculosis and leprosy (2). The increasing number of cases led the World Health Organization to consider BU as an emerging disease of major concern and to establish the BU global initiative in 1998.

BU histopathology is characterized by extensive areas of s.c. necrosis, with clumps of extracellular acid-fast bacilli (AFB) (3). These observations, in addition to others showing that, under some in vitro conditions: 1) *M. ulcerans* or its toxin mycolactone inhibits the phagocytic activity of macrophages (4–7); and 2) *M. ulcerans* fails to grow within macrophages (4, 7) and have led to the interpretation that *M. ulcerans* is an extracellular pathogen. However, as recently reviewed (8), early work on human and experimental *M. ulcerans* infections has shown that the BU agent is found within macrophages and neutrophils (9–12), an observation confirmed by recent work with mouse experimental infections (5, 13, 14) and in human BU samples (14). As the host cell of pathogenic mycobacteria is the macrophage (15, 16) the observation that *M. ulcerans* has a phase of intramacrophage proliferation (14) shows that this pathogen is an intracellular parasite like the other pathogenic mycobacteria. After an initial phase of intracellular proliferation, lasting for a period of time that depends on the strain cytotoxicity/virulence, *M. ulcerans* kills the host macrophage, becoming therefore extracellular (14).

The lysis of host cells and the distinctive tissue destruction observed in BU infection foci are associated with the production of mycolactone (17). Mycolactone was shown to passively diffuse through the plasmatic membrane of cells (18) inducing apoptosis and necrosis (4), having therefore an important impact on disease progression. This exotoxin induces cell death of the inflammatory infiltrate recruited during *M. ulcerans* infections (13, 14, 19) and inhibits the production of the macrophage-activating cytokine TNF (20, 21). Nevertheless, in some individuals, a protective immune response is mounted, ultimately controlling the pathogen without clinical manifestations (22). Additionally, patients with an already established infection can spontaneously heal (23). However, the characteristics of the protective immune response against *M. ulcerans* are not clarified.

Despite the fact that different authors have reported the expression of several cytokines in BU lesions, including the Th1 cytokine IFN-γ (24–27), there is no direct evidence for a protective

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Abbreviations used in this paper: AFB, acid-fast bacilli; BCG, bacillus Calmette-Guérin; BMDM, bone marrow-derived macrophage; BU, Buruli ulcer; cDMEM, complete DMEM; LRG-47, immunity-related GTPase family M member 1; MOI, multiplicity of infection; NOS, NO synthase; WT, wild-type.

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role of this cytokine in BU. IFN-γ, produced by T cells and NK cells (28, 29), is a key cytokine for the activation of the macrophage microbicidal mechanisms, leading to the control of intracellular infections such as tuberculosis (28, 29). Indeed, IFN-γ–activated macrophages show an increased fusion of phagosomes with endosomes and lysosomes, resulting in a decreased pH of the phagolysosomal compartment, which in turn, leads to *Mycobacterium tuberculosis* growth arrest (28). In addition, IFN-γ and TNF activate NO synthase (NOS)2 to produce NO, a potent microbicidal molecule (28). In IFN-γ–deficient mice, *M. tuberculosis* proliferation is not controlled (30, 31) and lack of macrophage activation and associated NOS2 expression are the likely factors that render these mice extremely susceptible to infection (30).

There is no direct evidence for a protective role of IFN-γ in BU. However, the recent demonstration of an intramacrophage growth phase for *M. ulcerans* (14) prompted us to investigate the role of IFN-γ in experimental mouse infections, as well as its effects on *M. ulcerans*-infected macrophages. Our results show that IFN-γ is expressed in *M. ulcerans*-infected tissues. Moreover, the increased bacterial proliferation in IFN-γ–deficient mice of the avirulent mycolactone-negative strain 5114 and, to a lesser extent, the intermediate virulent strain 94–1327, shows that IFN-γ confers some degree of protection against experimental BU. In vitro, IFN-γ–activated macrophages controlled the proliferation of *M. ulcerans* 5114 and *M. ulcerans* 94–1327 at low multiplicities of infection (MOIs) through a NO-dependent mechanism, associated with maturation and acidification of phagosomes. Induction of these microbicidal mechanisms was however absent in macrophages infected with the highly virulent strain 98–912. We show that this inhibition was mycolactone dependent, as intramacrophage control of the mycolactone-negative strain was reverted by the addition of increasing concentrations of mycolactone purified from strain 98–912.

These results are in accordance with previous data suggesting the involvement of cellular-mediated immune and delayed-type hypersensitivity responses in human and experimental BU (see *Discussion*) and provide, for the first time, a mechanism by which mycolactone modulates the macrophage intracellular environment to promote *M. ulcerans* proliferation.

Materials and Methods

Bacterial strains and growth conditions

The *M. ulcerans* strains used in this study were selected based on their cytotoxicity, virulence for mice and the type of mycolactone produced. Strain 98–912, a highly cytotoxic and virulent strain (13, 21), was isolated from a Chinese patient and produces a mycolactone D (32). Strain 94–1327, an intermediate cytotoxic and virulent strain (21), is an isolate from Australia and produces the characteristic mycolactone C (33), because its plasmid lacks the gene *MUP053* encoding a P450 hydroxylase required to hydroxylate the mycolactone side chain at C-12 to produce mycolactone A/B (34). Strain 5114, an avirulent strain (13, 21), is an isolate from Mexico and does not produce mycolactone (33) because of the loss of key genes involved in the synthesis of this macrolide (34). All strains used in this work are from the collection of the Institute of Tropical Medicine (Antwerp, Belgium).

The isolates were grown on solid 7H9 medium at 32°C for ~1 mo, recovered, diluted in PBS to a final concentration of 1 mg/ml and vortexed using 2-mm glass beads. The number of AFB in the inocula was determined according to the method described by Shepard and McRae (35). The final suspensions revealed >90% viable cells as assessed with LIVE/DEAD Baclight Kit (Molecular Probes, Leiden, The Netherlands).

Animals

Eight-week-old female BALB/c, C57BL/6, and IFN-γ–deficient mice in a C57BL/6 background were obtained from Charles River Laboratories (Barcelona, Spain) and housed in specific pathogen-free conditions with food and water ad libitum. The studies involving animals were reviewed and approved by the review committees of the Life and Health Sciences Research Institute and the Portuguese Governmental Agency *Direcção Geral de Veterinária*.

Footpad infection and bacterial growth

Mice were infected in the left hind footpad with 0.03 ml of *M. ulcerans* suspensions containing 5 $\log_{10}$ AFB. In all experiments, for ethical reasons, mice infected with the mycolactone-producing strains were sacrificed at the onset of ulceration.

At different time points postinfection, *M. ulcerans* growth in footpad tissues of infected mice was evaluated as described previously (13). Briefly, tissue specimens were carefully minced on a petri dish. The tissues were resuspended in 2 ml of PBS containing 0.04% Tween 80 (Sigma-Aldrich, St. Louis, MO) and vortexed vigorously with 2-mm glass beads to obtain homogenized suspensions. At different times postinfection, AFB counts were performed on samples from four footpads in each group by using the method of Shepard and McRae (35).

Extraction of cells from infected footpads

Infected footpads were collected and incubated in DMEM (Life Technologies, Paisley, U.K.) supplemented with 10 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Life Technologies), 10 mM glutamine (Life Technologies), and 125 U/ml collagense XI (Sigma-Aldrich) for 2 h at 37°C. Each footpad was filtered through a 40-μm nylon cell strainer and washed with DMEM with 10% heat-inactivated FBS (Sigma-Aldrich). Samples from four infected mice were pooled for mRNA isolation and quantitative RT-PCR analysis.

Culture of murine bone marrow-derived macrophages

Macrophages were derived from the bone marrow as follows: mice were euthanatized with CO_2 and femurs removed under aseptic conditions. Bones were flushed with 5 ml of cold HBSS (Life Technologies). The resulting cell suspension was centrifuged at $500 \times g$ and resuspended in DMEM with 10% L929 cell-conditioned medium (complete DMEM [cDMEM]). To remove fibroblasts or differentiated macrophages, cells were cultured for a period of 4 h on cell culture dishes (Nunc, Naperville, IL) with cDMEM. Nonadherent cells were collected with warm HBSS, centrifuged at $500 \times g$, distributed in 24-well plates at a density of 5×10^5 cells/well, and incubated at 37°C in a 5% CO_2 atmosphere. On day 4 after seeding, 0.1 ml of L929 cell-conditioned medium was added, and medium was renewed on the seventh day. After 10 d in culture, cells were completely differentiated into macrophages. Twelve hours before infection, macrophages were incubated at 32°C in a 5% CO_2 atmosphere and maintained until the end of the experimental infection as described elsewhere (13).

Macrophage infectivity assays and bacterial growth

Bacterial suspensions were prepared as described above and further diluted in cDMEM before infecting macrophage monolayers. *M. ulcerans* suspensions (0.2 ml) were diluted in cDMEM and added to each well to obtain the MOI indicated for each experiment (bacteria to macrophage ratio). Cells were incubated for 4 h at 32°C in a 5% CO_2 atmosphere and then washed four times with warm HBSS to remove noninternalized bacteria and reincubated in cDMEM alone or supplemented with 100 U ml^{-1} rIFN-γ (R&D Systems, Minneapolis, MN), in the presence or not of N^G-monomethyl-L-arginine, an inhibitor of NOS, or N^G-monomethyl-D-arginine, a molecule that does not have an effect in the function of NOS. To confirm the MOI, counting of AFB in infected macrophages was performed at the beginning of experimental infection as described previously (35).

At different time points postinfection, bacterial growth was assessed by CFUs counting from macrophage monolayers. Briefly, macrophage monolayers were lysed with saponin (0.1% final concentration), and serial dilutions were seeded in 7H9 agar medium with oleic acid-albumin-dextrose complex supplement. Plates were then incubated at 32°C for 6–8 wk.

Purification of mycolactone from M. ulcerans *98–912*

Mycolactone D was isolated from midlog phase cultures of *M. ulcerans* 98–912 grown in Dubos medium supplemented with 10% oleic acid-albumin-dextrose complex and incubated at 32°C, as described previously (33). Briefly, bacteria were harvested by centrifugation, and lipids were extracted with chloroformmethanol (2:1, v/v) for 4 h. The organic phase was separated by the addition of a 0.2 volume of water, centrifuged, and dried in a rotary evaporator. The remaining lipids were resuspended in ice-cold acetone to precipitate phospholipids. The individual lipid components of the acetone-soluble lipid fraction were separated by preparative chromatography by using the CycloGraph instrument (Analtech, Newark, DE). The separated

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fractions were further analyzed by TLC, and the fractions corresponding to mycolactone D were confirmed by mass spectrometry (MS detector Thermo LxQ linear ion trap). The fractions containing purified mycolactone were pooled, resuspended in absolute ethanol, and stored at 4°C.

In some in vitro experiments of bone marrow-derived macrophage (BMDM) infection, mycolactone D purified as described above was added to the infected monolayers at different concentrations. The supernatants were assayed for nitrite, and the RNA extracted from adherent macrophages for real-time PCR analysis.

Nitrite quantification

Nitrite production by macrophage monolayers was determined by the Griess assay as described elsewhere (36). Briefly, the supernatants recovered from the macrophage infectivity assays were diluted in a 96-well ELISA plate (Nunc) with an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine, and 2.5% H_3PO_4). The absorbance at 550 nm was measured, and the concentration of nitrite was calculated by comparing OD values to a standard curve of $NaNO_2$ in cDMEM.

Lysotracker-Red staining of infected BMDM

BMDM were infected for a four hour period with the *M. ulcerans* strains previously stained with SYTO 9 (Molecular Probes), according to the manufacturer's instructions. To label acidic cellular compartments, Lysotracker Red DND-99 (Molecular Probes) was added to the culture wells at selected time points to a final dilution of 1/20,000 and incubated for 20 min at 32°C in a 5% CO_2 atmosphere. After this, BMDM were washed with PBS and fixed with 4% formaldehyde at room temperature. Confocal microscopy images were acquired as Z-stacks with 0.2–0.3 mm spacing using a ×60 1.42 NA on an Olympus FluoView FV1000 confocal microscope. Maximal intensity projections of the entire Z-stack are shown.

Quantitative RT-PCR analysis

Total RNA from dermal cells extracted from infected footpads and cultured BMDM was extracted with TRIzol reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reverse transcription was done with whole RNA in a final volume of 20 μl using SuperScript II (Invitrogen) and Oligo(dT) (Invitrogen), according to the manufacturer's instructions. The cDNA was then subjected to real-time PCR for quantification of hypoxanthine phosphoribosyltransferase, IFN-γ, and immunity-related GTPase family M member 1 (LRG-47). The LightCycler-FastStart DNA Master Hybridization Probes mixture was used according to the manufacturer's instructions. The hypoxanthine phosphoribosyltransferase reaction mixture included 5 mM Mg^{2+}, sense primer (5′-GCT GGT GAA AAG GAC CTC T-3′), antisense primer (5′-CAC AGG ACT AGA ACA CCT GC-3′), and the gene-specific probes (5′-LC red640-TCT GCA AAT ACG AGG AGT CCT GTT G_P-3′ and 5′-AAA GCC TAA GAT GAG CGC AAG TTG A_F-3′). The cycling parameters were 1 cycle of 95°C for 10 min, followed by 45 cycles of 94°C for 10 s, 60°C for 10 s, and 72°C for 11 s. The temperature transition rate was 20°C. The IFN-γ reaction mixture included 4 mM Mg^{2+}, sense primer (5′-TGG CAA AAG GAT GGT GAC ATG-3′), antisense primer (5′-GAC TCC TTT TCC GCT TCC TGA-3′), and the gene-specific probes (5′-$_{LCred640}$-TCC AGC GCC AAG CAT TCA ATG AGC-$_P$-3′ and 5′-TGC CAA GTT TGA GGT CAA CAA CCC ACA-$_F$-3′). One cycle of 95°C for 10 min, followed by 45 cycles of 94°C for 10 s, 52°C for 10 s, and 72°C for 12 s The LRG-47 reaction mixture included 5 mM Mg^{2+}, sense primer (5′-CTC TGG ATC AGG GTT TGA GGA GTA-3′), antisense primer (5′-GGA ACT GTG ATG GTT TCA TGA TA-3′), and the gene-specific probes (5′-LC red640- AGG TCC ACA GAC AGC GTC ACT CGG_P-3′ and 5′-AAC CAG AGA GCC TCA CCA GGG AGC TGA_F-3′). The cycling parameters were 1 cycle of 95°C for 10 min, followed by 45 cycles of 94°C for 10 s, 58°C for 10 s, and 72°C for 20 s

The specific probes for each cytokine were designed and synthesized by TIB MolBiol (Berlin, Germany). Single acquisition was done in the end of each annealing step.

Statistical analysis

Statistical significance of values was determined using the Student *t* test.

Results

Expression of IFN-γ during infection by M. ulcerans *plays a role in the control of bacterial growth*

IFN-γ has been shown to play a major role in the control of infections by intracellular pathogens, including *Mycobacterium avium* (37, 38) and *M. tuberculosis* (30, 31). Although IFN-γ has been suggested to be associated with resistance to BU (24, 25, 27), the role of this cytokine in *M. ulcerans* infections has not been experimentally addressed. We started by investigating whether IFN-γ was expressed following *M. ulcerans* infection. For this, groups of mice were infected in the footpad with three strains of *M. ulcerans* showing different degrees of virulence, according to the absence or presence of different types of mycolactone: *M. ulcerans* 98–912 produces mycolactone D and is highly virulent (13, 33); strain 94–1327 produces mycolactone C and shows intermediate virulence (21, 34) and; *M. ulcerans* 5114 is an avirulent, mycolactone-deficient mutant strain (13, 34). All the tested strains induced the expression of IFN-γ at the site of infection, with a peak of expression observed between days 20 and 30 postinfection (Fig. 1), followed by a decrease in message expression. Interestingly, the highly virulent strain 98–912 induces a 1.9-fold higher level of IFN-γ mRNA compared with the avirulent strain 5114 and a 1.5-fold increase compared with the intermediate virulent strain 94–1327. Although it has been suggested that susceptibility to BU is associated with the development of a Th2 type of response (39), we did not detect an increase in the expression of IL-4 in the footpad throughout the experimental period of infection regardless the virulence of the strain (data not shown) as previously reported in BU patients (26).

These results show that, as in human BU lesions (24, 25, 27), IFN-γ is expressed in the infection foci of mice infected with *M. ulcerans*. Next, we decided to investigate the biological activity of this cytokine in experimental infections by comparing the proliferation of the different *M. ulcerans* strains in footpads of IFN-γ–deficient mice or wild-type (WT) counterparts. As shown in Fig. 2*A*, the proliferation of *M. ulcerans* 5114 was controlled by WT mice with induction of bacteriostasis, but not by their IFN-γ-deficient counterparts, with a 1.5 $\log_{10}$ increased AFB counts at day 245 postinfection. IFN-γ–deficient mice also showed an increased, although transient, susceptibility to infection with the intermediate virulent strain 94–1327, with an augmented proliferation of 0.4 $\log_{10}$ AFB over the first 35 d of experimental infection (Fig. 2*B*). After this time point, this strain was not controlled by either WT or IFN-γ–deficient mice. In contrast, no differences were found in the proliferation of the highly virulent strain *M. ulcerans* 98–912 (Fig. 2*C*).

The fact that IFN-γ is expressed in tissues infected with the highly virulent strain (Fig. 1*C*) in the absence of bacterial control suggests an inhibition of the protective activity of this cytokine on macrophages infected with *M. ulcerans* 98–912.

The protective role of IFN-γ during infections with M. ulcerans *5114 or 94–1327 is associated with macrophage activation*

To further address the role of IFN-γ in infections by *M. ulcerans*, cultures of BMDM were infected with the selected *M. ulcerans* strains, in the presence or absence of exogenously added IFN-γ. As shown in Fig. 3*A*, *M. ulcerans* 5114 was not controlled by BMDM over the 8-d period studied. As expected from the in vivo results obtained for this strain (Fig. 2*A*), when infected macrophages were activated with IFN-γ, there was a 1.4 $\log_{10}$ reduction of the CFU counts (Fig. 3*A*). On the other hand, no differences were found in the proliferation of the mycolactone-producing strains between IFN-γ–activated or nonactivated macrophages at an MOI of 1:1 (data not shown). However, at a lower MOI of 1:3, IFN-γ–activated BMDM partially controlled the proliferation of the intermediate virulent *M. ulcerans* 94–1327, with a 0.4 $\log_{10}$ reduction of the CFU counts at day 4 postinfection and a 0.3 $\log_{10}$ reduction at day 8 postinfection (Fig. 3*B*). This bacterial control was not observed for the highly virulent strain 98–912 even at this low MOI (Fig. 3*C*). This effect is not associated with an early

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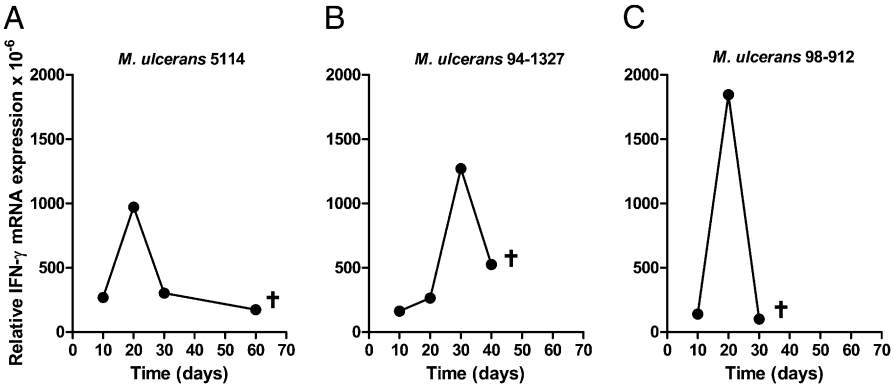

FIGURE 1. IFN-γ is expressed in *M. ulcerans*-infected footpads. C57BL/6J mice were infected s.c. with 5 $\log_{10}$ of *M. ulcerans* 5114 (*A*), 94–1327 (*B*), or 98–912 (*C*) in the left hind footpad. At different times postinfection, cells from the footpad were harvested; mRNA extracted and IFN-γ expression evaluated by real-time PCR. Results are from one representative experiment of three independent experiments, each with four pooled animals per group and time point. †Mice were sacrificed before the onset of ulceration.

death of macrophages, because we have previously demonstrated that at an MOI of 1:3, *M. ulcerans* 98–912 does not significantly compromise the viability of the macrophages during the phase of intracellular multiplication (14).

Overall, our results demonstrate that IFN-γ may activate macrophages to control the intracellular proliferation of *M. ulcerans*. However, the fact that the proliferation of mycolactone-positive strains is not controlled at high MOI suggests a previously unappreciated role of mycolactone in the inhibition of the IFN-γ–dependent macrophage microbicidal mechanisms. Therefore, we next sought to determine the macrophage microbicidal mechanisms required to kill intracellular *M. ulcerans* as well as the role of mycolactone in the modulation of these mechanisms.

IFN-γ–activated macrophages infected with M. ulcerans *5114 or 94–1327, but not* M. ulcerans *98–912, show an increased production of NO and phagosome maturation*

IFN-γ has been shown to increase the bactericidal activity of macrophages, namely by inducing phagosome-lysosome fusion (40) and NO production (28, 41). However, the relevance of these effector mechanisms for the control of *M. ulcerans* is not known. To investigate this question, we first measured the expression of LRG-47, a member of the 47-kDa GTPase family involved with phagosome maturation (42). As shown in Fig. 4*A*, IFN-γ–activated BMDM infected with *M. ulcerans* 5114 showed high levels of LRG-47 expression 12 h postinfection, an indicator of phagosome maturation. Importantly, the expression of LRG-47 in *M. ulcerans* 98–912-infected macrophages activated with IFN-γ was abrogated to levels similar to those of nonactivated infected macrophages (Fig. 4*A*). On the other hand, IFN-γ–activated macrophages infected with the intermediate virulent strain 94–1327 showed an intermediate expression of LRG-47 (Fig. 4*A*). In accordance with the results of LRG-47 expression, confocal colocalization studies showed that both *M. ulcerans* 5114 and 94–1327 were mostly found in acidified phagosomes, whereas the highly virulent *M. ulcerans* 98–912 bacteria were mainly observed in nonacidified cellular compartments (Fig. 4*B*).

This effect of IFN-γ on the acidification of *M. ulcerans*-containing phagosomes prompted us to evaluate the production of nitrite by infected macrophages. As depicted in Fig. 5*A*, IFN-γ–activated macrophages infected with the mycolactone-negative strain 5114 produced high amounts of nitrite over the 8 d of experimental infection. In contrast, no relevant NO production was observed in macrophages infected with the highly virulent strain 98–912 (Fig. 5*C*). At the low MOI of 1:3, *M. ulcerans* 94–1327-infected macrophages, in the presence of IFN-γ, produced nitrite at similar levels as those produced by macrophages infected with the mutant strain 5114 (Fig. 5*B*). To evaluate the relevance of the produced NO for the intramacrophage control of *M. ulcerans*, BMDMs were infected with strain 5114 in the presence of N^G-monomethyl-L-arginine, a competitive NOS inhibitor. As shown in Fig. 5*D*, the inhibition of NOS considerably reduced the capacity of IFN-γ–activated macrophages to control this strain.

Taken together, these results suggest that phagosome maturation and NO production are mechanisms by which macrophages control intracellular *M. ulcerans* and that these mechanisms are targeted by mycolactone, thereby providing a nonacidic environment, appropriate for the survival and proliferation of *M. ulcerans* within the host cell. This inhibition appears to be dependent on the type and/or amount of accumulated mycolactone.

Mycolactone impairs the IFN-γ–dependent microbicidal mechanisms of macrophages during infections by M. ulcerans

So far, our data suggest that highly virulent strains of *M. ulcerans* modulate the intramacrophage environment through the production of mycolactone, thus preventing a bactericidal activity of

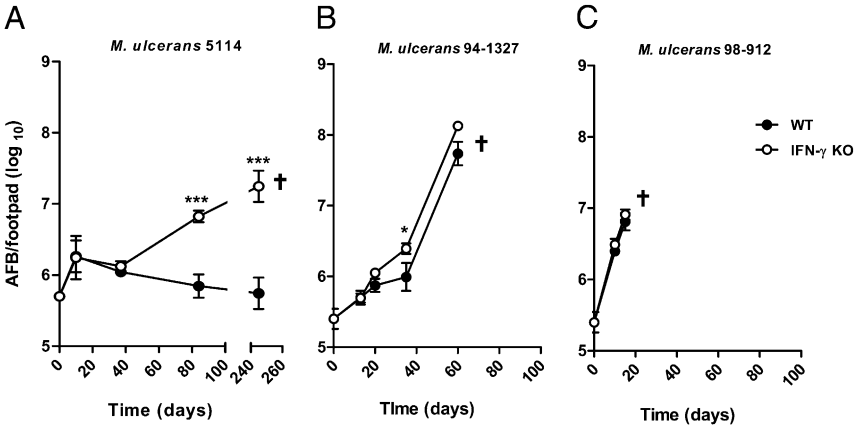

FIGURE 2. IFN-γ is protective during in vivo infections with *M. ulcerans* 5114 or 94–1327. C57BL/6J (●) and IFN-γ–deficient (○) mice were s.c. infected in the footpad with ~5.6 $\log_{10}$ AFB of *M. ulcerans* 5114, 94–1327, or 98–912. The number of AFB in homogenates of footpads from each group was counted at the indicated time points. Statistical differences were determined by using Student *t* test (*$p < 0.05$; ***$p < 0.001$). Results are from one representative experiment of two independent experiments each with at least three animals per group and time point. †Mice were sacrificed before the onset of ulceration.

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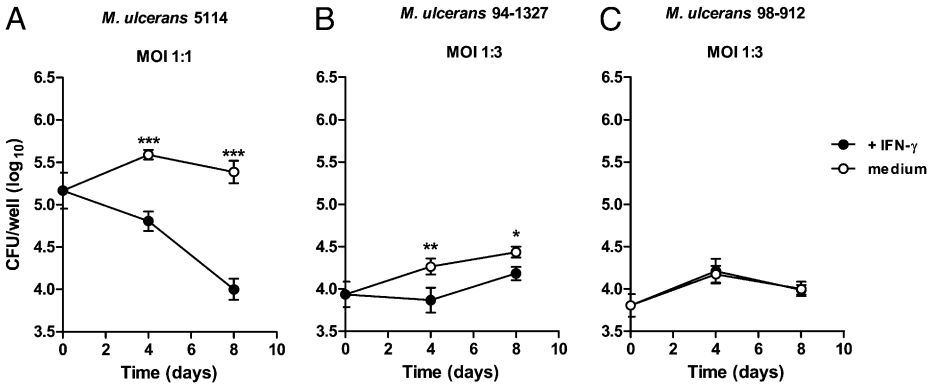

FIGURE 3. IFN-γ–activated macrophages control the proliferation of *M. ulcerans* 5114 or 94–1327. BMDM from BALB/c mice were infected at different MOI with *M. ulcerans* 5114 (*A*), 94–1327 (*B*), or 98–912 (*C*). Macrophages were left untreated (○) or were activated with 100 U/ml rIFN-γ (●). At different times postinfection, macrophages were lysed, and CFU count was performed. Statistical significance was determined by using Student *t* test ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). Results are from one representative experiment of three independent experiments.

macrophages. To confirm this hypothesis, cultures of BMDM were infected with *M. ulcerans* 5114 and supplemented with noncytotoxic concentrations (Ref. 21 and data not shown) of mycolactone D purified from strain 98–912. We decided to use this type of mycolactone instead of mycolactone C given the higher cytotoxicity of *M. ulcerans* 98–912 toward BMDM, as compared with *M. ulcerans* 94–1327 (21). As depicted in Fig. 6, addition of mycolactone to IFN-γ–activated, *M. ulcerans* 5114-infected macrophages had an inhibitory effect in the expression of LRG-47 and NO production. This effect was clearly dose dependent, being the expression of LRG-47 and the production of NO abrogated at high mycolactone doses (Fig. 6*A*, 6*C*). In line with this, bacteria were found in nonacidic compartments, when infected macrophages were cultured with 25 ng of mycolactone, contrary to control-infected macrophages (Fig. 6*B*). Finally, we showed that in the presence of low concentrations of mycolactone isolated from the highly virulent strain, bacteria were found in acidic compartments (Fig. 6*B*), and macrophages were still able to control *M. ulcerans* proliferation (Fig. 6*D*), even though LRG-47 expression and NO production were already reduced (Fig. 6*A*, 6*C*). This control was lost at higher concentrations of mycolactone, when both LRG-47 and NO production were abrogated (Fig. 6*D*).

These results show that mycolactone produced by *M. ulcerans* plays a key role in the impairment of the macrophage microbicidal activities by inhibiting the production of NO and modulating the phagosomal environment, thereby allowing *M. ulcerans* intramacrophage proliferation.

Discussion

Mycolactone plays a key role in the pathology induced by *M. ulcerans* infections, both in humans and experimental animal models (10, 11, 13, 43). Additionally, in some vitro conditions, mycolactone induces cell death (44) and inhibits *M. ulcerans* uptake by phagocytes (4, 5, 7), which led to the interpretation that *M. ulcerans* was an extracellular pathogen (4, 17, 43, 45–47). However, we have recently described an intramacrophage growth phase for *M. ulcerans* (14), showing that although apparently contradictory, the production of a cytotoxic exotoxin can be conciliated with an intracellular lifestyle. These observations are in accordance with the cellular-mediated immune and delayed-type hypersensitivity responses induced in BU patients postinfection and required for *M. ulcerans* control (9, 24, 26, 39, 46, 48–51). The host immune response is therefore determined by the

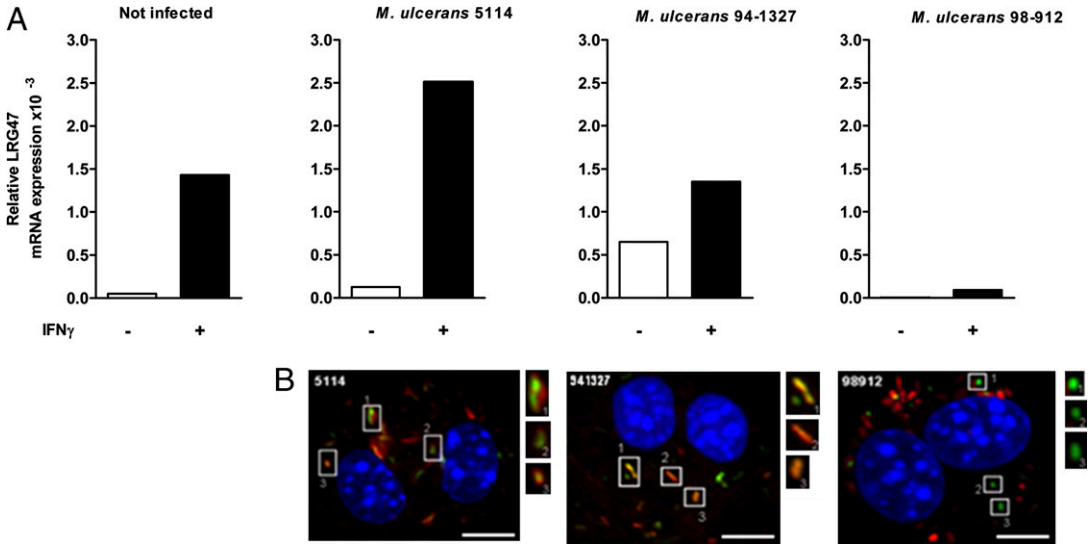

FIGURE 4. Phagosome maturation and acidification are induced during infection with *M. ulcerans* 5114 or 94–1327. *A*, BMDM from BALB/c mice infected with *M. ulcerans* 5114, 94–1327, or 98–912 were either left untreated (□) or were activated with 100 U/ml rIFN-γ (■). At 12 h postinfection, mRNA was extracted, and LRG-47 expression was evaluated by real-time PCR. *B*, BMDM were infected with fluorescent-labeled *M. ulcerans* 5114, 94–1327, or 98–912 (shown in green) in the presence of 100 U/ml rIFN-γ. Twenty-four hours postinfection, BMDM were labeled with Lysotracker Red DND-99 to specifically stain acidified compartments (shown in red) and DAPI to stain nuclei (shown in blue). Confocal microscopy images were acquired as Z-stacks and maximal intensity projections of the entire Z-stack are shown. Bars, 5 μm (magnification ×1500). Results are from one representative experiment of two independent experiments.

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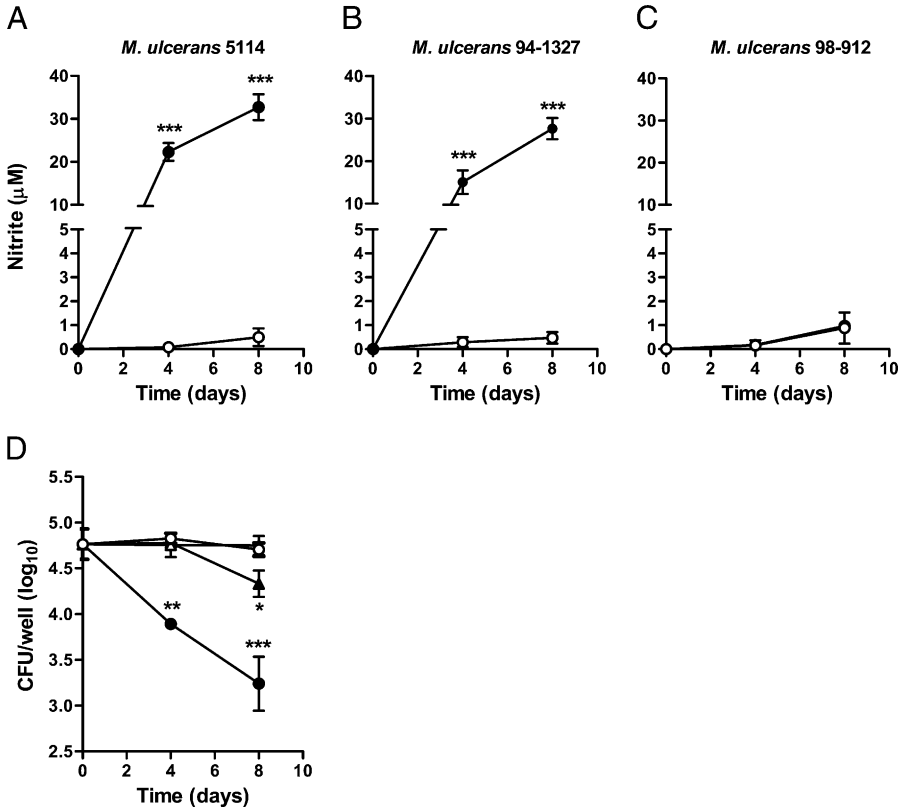

FIGURE 5. Nitrite produced by IFN-γ–activated macrophages are involved in the control of *M. ulcerans* proliferation. To assess nitrite production, BMDM from BALB/c mice were infected at an MOI 1:1 with *M. ulcerans* 5114 (*A*) or at an MOI 1:3 with strains 94–1327 (*B*) or 98–912 (*C*). Macrophages were either left untreated (○) or were activated with 100 U/ml rIFN-γ (●). At different time points, the levels of nitrite production were assessed in the culture medium. Results are from one representative experiment of three independent experiments. *D*, To determine the protective role of the produced nitrite, BMDM were infected with *M. ulcerans* 5114 at an MOI of 1:1 in the absence (○) or presence of 100 U/ml rIFN-γ (●). Cultures were supplemented with the competitive inhibitor N^G-monomethyl-L-arginine (▲) or, as a control, with N^G-monomethyl-D-arginine (circles). Results are from one representative experiment of three independent experiments. Nitrite production was not detected in noninfected BMDM, activated or not with IFN-γ (data not shown). Statistical significance was determined by using Student *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

intracellular nature of this mycobacterial pathogen compounded with the cytotoxic activity of an exotoxin.

Furthering the understanding on the immune response against *M. ulcerans*, we show in this study for the first time that the IFN-γ–dependent activation of macrophages is required for the host control of this *Mycobacterium*, as shown by in vitro and in vivo models of infection. In fact, IFN-γ–deficient mice are more susceptible to infections by the avirulent strain 5114 and, to a lesser extent, to the intermediate virulent, mycolactone-producing *M. ulcerans* 94–1327. However, for the highly virulent strain 98–912, no differences were found between IFN-γ–deficient mice and WT counterparts, irrespective of the infectious dose, thus suggesting that mycolactone impairs the protection conferred by this cytokine. Our in vitro findings show that indeed mycolactone modulates the macrophage microbicidal activity triggered by IFN-γ through the inhibition of phagosome maturation and NO production. Thus, by inhibiting the activation of the macrophage microbicidal mechanisms, mycolactone gives a selective advantage to *M. ulcerans*, preventing its killing by the host cell before it induces the lysis of the infected macrophages, becoming therefore extracellular to infect other cells (14). In addition to the effect of mycolactone, and as recently reviewed (8), other factors are likely associated with the pathogenicity of *M. ulcerans*, which may contribute to the inhibition of phagosome maturation and macrophage activation. Indeed, it has been shown that the ability of *M. tuberculosis* to grow inside macrophages is partially dependent on its capacity to prevent phagosome maturation, although the mechanisms for this inhibition are not completely understood (16, 28).

Our findings are in line with several studies with human BU patients suggesting that IFN-γ is associated with protection (24, 27, 39, 49). This cytokine was firstly suggested to play a role in the control of *M. ulcerans* infections by Gooding et al. (24, 39), who report that PBMCs from patients with past BU, but not from healthy contacts, display low capacity to produce IFN-γ after stimulation with live *M. ulcerans* or *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). In a posterior study, Westenbrink et al. (51) reported that purified protein derivative-stimulated PBMCs from BU patients produce significantly higher levels of IFN-γ in late stages of the disease but not in early stages, as compared with matched community controls. These data were recently confirmed (52). When PCR analysis was used to quantify the expression of this cytokine in nodular or ulcerative BU lesions, Prévot et al. (26) reported a higher expression of IFN-γ in the nodular lesions while a lower expression was detected in the more severe ulcerative lesions. On the other hand, in a more recent study, Phillips et al. (27), using a higher number of patients, reported a strong expression of IFN-γ in both nodular and ulcerative lesions. Also in line with our findings, supporting therefore the existence of protective cellular-mediated immune and delayed-type hypersensitivity responses, associated with IFN-γ production in the context of Th1 responses, when BU disease progresses to healing, granuloma formation occurs (3, 46, 53–55), and the positivity of the Burulin skin test increases (56–58). In addition, BCG vaccination was found to protect humans against BU osteomyelitis (59), and BCG or a DNA vaccine encoding Ag85A from BCG confer some degree of protection against *M. ulcerans* experimental infections (60, 61). We have also analyzed the expression of IL-10 in infected footpads, observing an increased mRNA level at later time points during infection with the highly virulent strain 98–912 (data not shown), coinciding with the sudden decrease found in the expression of IFN-γ. However, because, at this stage, there is already substantial damage of the infected tissue (13), it is difficult to dissociate whether the induction of IL-10 works as a counterregulatory mechanism induced by *M. ulcerans* or whether it is a result of an internal regulation associated to the massive tissue necrosis.

The reports discussed above suggest a protective role for IFN-γ in BU. We have experimentally confirmed this hypothesis in the current study by showing that IFN-γ–dependent phagosome maturation and NO production are required to control the

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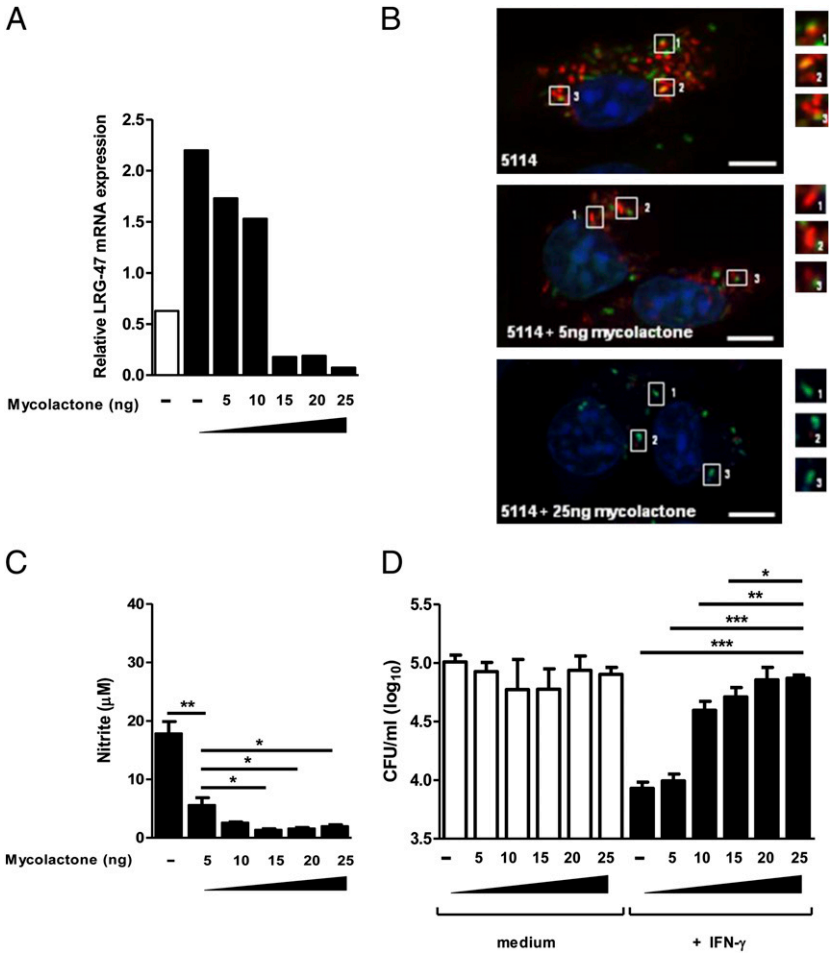

FIGURE 6. Mycolactone dose-dependent inhibition of the IFN-γ–mediated protection in *M. ulcerans*-infected macrophages. BMDM cultures from BALB/c mice were infected with the mycolactone-negative *M. ulcerans* 5114 at an MOI of 1:1 and supplemented with 100 U/ml rIFN-γ (■) or not (□). Macrophages were either left untreated or treated with different concentrations of mycolactone D isolated from strain 98–912. *A*, At 24 h postinfection, cells were recovered for mRNA extraction, and the expression of LRG-47 was evaluated by real-time PCR. *B*, For confocal microscopy imaging, macrophage cultures were infected with fluorescent-labeled *M. ulcerans* 5114 (shown in green) and supplemented with 100 U/ml rIFN-γ in the absence or presence of 5 or 25 ng of mycolactone. Twenty-four hours postinfection, BMDM were labeled with Lysotracker Red DND-99 to specifically stain acidified compartments (shown in red) and DAPI to stain nuclei (shown in blue). Images were acquired as Z-stacks and maximal intensity projections of the entire Z-stack are shown. Bars, 5 μm (magnification ×1500). *C*, The levels of nitrite were measured in the culture medium 4 d postinfection. *D*, To determine the impact of mycolactone on the macrophage capacity to control *M. ulcerans* growth, infected cells were lysed, and CFU performed 4 d postinfection. Nitrite production was not detected in noninfected BMDM, activated or not with IFN-γ (data not shown). Statistical significance was determined by using Student *t* test ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). Results are from one representative experiment of three independent experiments.

intracellular proliferation of *M. ulcerans*. The importance of NO for the control of infections by *M. ulcerans* or by *M. tuberculosis* (30) contrasts with that of infections by other mycobacteria such as *Mycobacterium avium* for which NO is not relevant (62). Supporting our observation is the fact that, such as for *M. tuberculosis*, *M. ulcerans* is sensitive to acidified nitrites (63). On the other hand, our observations in the mouse model do not support that a shift from a Th1 to a Th2 type of response is associated with susceptibility to *M. ulcerans* infections, which is in line with a previous report on BU patients (26). The occurrence of additional host protective antimicrobial mechanisms, dependent or not on IFN-γ, cannot be excluded at the present stage and could either act during the intracellular growth phase of *M. ulcerans* or after its shedding from the infected cells.

Mycolactone was suggested to have an inhibitory effect on the production of different cytokines and chemokines, namely MIP-1α, MIP-1β, RANTES (CCR5 ligands), and inflammatory protein-10 (CXCR3 ligand) by cultured dendritic cells (64). Although the impact of mycolactone on the expression of these chemokines could interfere with the homing capacity of IFN-γ–secreting T cells to infected tissues (64), this has not been definitively demonstrated in vivo. Our data, however, shows that IFN-γ is expressed in *M. ulcerans*-infected tissues, irrespective the secretion of mycolactone by the infecting strain, suggesting that mycolactone does not completely inhibit the homing of IFN-γ–producing cells in all the phases of the infectious process. However, in the mouse model, this initial response is clearly not enough for the establishment of protection against the highly virulent strain of *M. ulcerans*. The absence of protective immune responses against infections by mycolactone-producing strains could be explained by the accumulation of high concentrations of toxin at advanced stages of the infectious process, which would impair macrophage activation, as suggested by our present results, and/or induce the cell death of incoming and resident leukocytes (13, 14). However, the exact concentration at the infection focus of this poorly immunogenic, lipidic toxin is not known, because its detection by immunohistochemistry has not been possible. It was recently reported, in mice s.c. infected with *M. ulcerans*, that the intact mycolactone molecule was present in mononuclear cells of blood, lymph nodes, and spleen, as detected by mass spectrometry analysis (65). However, the technical approach used in that study did not allow the assessment of the distribution, concentration, and biological activity of mycolactone at the *M. ulcerans*-infected s.c. sites.

Our present results clarify an important aspect of the immune response against *M. ulcerans* and might also be relevant for future vaccine studies. If an early arrival of IFN-γ–producing T cells to the infection foci is promoted through previous vaccination, one can speculate that the proliferation of mycolactone-producing *M. ulcerans* strains could be halted before accumulation of high toxin concentrations. Our data can also explain the effects observed in patients treated with the combination of Rifampicin and Streptomycin, during which immune activation has been suggested to be required to achieve sterilization. In fact, the antibiotic administration has been shown to increase phagocytosis of bacilli at the infection foci and to promote inflammatory cellular responses (66), associated with an increased production of IFN-γ (67), which can be related to the reduction of mycolactone concentrations, allowing macrophage activation and further containment of the infection. Accordingly, it has been reported that after the

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surgical excision of BU lesions, the IFN-γ production is increased (68). IFN-γ may also be involved in the spontaneous healing of lesions that occurs in BU patients (3, 23).

In summary, our results show for the first time that the IFN-γ–dependent phagosome maturation and NO production are mechanisms required for the control of *M. ulcerans*. Mycolactone was shown to play a key inhibitory role in this process, although the precise mechanism by which this inhibition is achieved needs further clarification. This set of results is highly relevant for future vaccine design and for the development of new strategies of antimicrobial therapy.

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Disclosures

The authors have no financial conflicts of interest.

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