Universidade do Minho
Escola de Ciências da Saúde

Egídio Manuel Pires Torrado

Cellular immune response to experimental infections with mycobacteria with different degrees of virulence: development of new preventive strategies

Estudo da resposta imune celular à infecção experimental por micobactérias de diferentes virulências: desenvolvimento de novas estratégias preventivas

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Trabalho efectuado sob a orientação de:
Doutor Jorge Manuel Rolo Pedrosa
Professor Associado da Escola de Ciências da Saúde,
Universidade do Minho, Braga, Portugal

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DECLARAÇÃO

Nome: Egídio Manuel Pires Torrado

Endereço electrónico: egidio@ecsaude.uminho.pt

Telefone: +351 933198841

Número do Bilhete de Identidade: 10819423

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Orientador:

Doutor Jorge Manuel Rolo Pedrosa

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Aos meus pais,
que sempre acreditaram em mim...

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ABSTRACT

Mycobacterium ulcerans is the etiological agent of Buruli ulcer (BU), a serious tropical, necrotizing skin disease that can cause terrible deformities and disabilities if not treated at early stages. This pathogen was first identified in 1948 by MacCallum and colleagues in Australia; however, until the last decade, BU has received little attention from the scientific community.

BU affects mainly rural and poor communities in Africa. Different control measures have been suggested, however, associated with BU, there is a strong social stigma, and BU is often seen as a curse rather than a disease. Thus, the development of a specific vaccine would be the most satisfactory strategy to control BU. It is therefore critical to advance the knowledge on the host/parasite interactions concerning this enigmatic disease.

The pathology of BU is closely associated with mycolactone, a polyketide exotoxin produced by *M. ulcerans* and which is the major virulence factor of the agent of BU. It is known that cell wall-associated mycolactone or free toxin that diffuses across the tissues, induces apoptosis and necrosis of cells, and, ultimately, the formation of ulcers. Indeed, tissue necrosis and extracellular clumps of *M. ulcerans* bacilli are the histopathological hallmark of BU. The extensiveness of necrotic areas devoid of cells, which expand, possibly due to mycolactone diffusion, beyond areas where *M. ulcerans* bacilli can be found, has lead to the assumption that *M. ulcerans* induces low or absent cellular inflammatory responses. Additionally, the presence of large clumps of free bacteria in the central acellular areas directed early investigators to classify *M. ulcerans* as an extracellular pathogen, an exception among pathogenic mycobacteria. In contrast, many reports have described the occurrence of cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH) responses in BU patients, strongly suggesting that macrophages play an effector role in the control of infection.

The use of an animal model of infection, in which it is possible to analyze the entire lesion at very early time points, is essential to understand the interaction of *M. ulcerans* with the host immune cells, particularly phagocytes. Previous work from our laboratory in the mouse footpad model of infection has shown that inoculation of *M. ulcerans* in the subcutaneous tissue induces an inflammatory cellular response with the involvement of macrophages and neutrophils. Moreover, our group has shown that even in advanced lesions, inflammatory cells are consistently present, although restricted to the periphery of the necrotic infectious center.

The constant availability of phagocytes to interact with *M. ulcerans*, at the persistent areas of inflammatory cellular infiltration, directed me to reevaluate the interaction of this pathogenic mycobacterium with the host macrophages, in the context of my PhD thesis, both in human and in experimental infections.

In experimental murine infections, we showed that *M. ulcerans* bacilli were phagocytosed in the subcutaneous tissue early after infection. At later time points, as previously described, we found free *M. ulcerans* organisms concentrated in central necrotic acellular areas. Surrounding these necrotic areas, where inflammatory infiltrates could be seen, *M. ulcerans* bacilli were however found mainly within macrophages. A histological evaluation of serial sections of BU tissue samples from 15 African patients with inflammatory infiltrates, confirmed the occurrence in 8 patients of intracellular bacilli within phagocytes infiltrating the peripheral areas. In vitro, at low multiplicities of infection (MOI), we found that *M. ulcerans* was efficiently phagocytosed by macrophages, at similar rates as those for the intracellular pathogens *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Within macrophages, *M. ulcerans* was found surrounded by the phagosomal membrane in tight or spacious phagosomes. We also demonstrated that both virulent, mycolactone-producing, as well as mutant, nonproducing strains grew inside cultured macrophages when low MOIs were used to prevent mycolactone-associated cytotoxicity. After this initial phase of intracellular residence and proliferation, mycolactone-producing strains were found to lyse the macrophage, becoming extracellular. These results suggest that *M. ulcerans* is an intracellular pathogen like the other virulent mycobacteria, which explains the reported occurrence of CMI and DTH in model and human cases of *M. ulcerans* infection.

Following the demonstration of an intramacrophage growth phase for the agent of BU, our work focused on the role played by cytokines that are associated with the host resistance to intracellular pathogens.

Tumor necrosis factor (TNF) and interferon-gamma (IFN-γ) are two cytokines with a key role in the activation of macrophages, required to the control of intracellular mycobacteria. A previous report showed that a lipidic fraction of *M. ulcerans* culture filtrates containing mycolactone suppressed the production of TNF by cultured human monocytes. On the other hand, the expression of TNF has been reported in BU lesions. Therefore, we considered pertinent to study the effect of the infection with different strains of *M. ulcerans* on the

production of this cytokine by macrophages, as well as to assess in vivo the role played by TNF in infections by *M. ulcerans*.

We confirmed previous observations of other authors by showing that mycolactone inhibits TNF production, in a dose-dependent manner, and we have expanded these observations by using a model of primary mouse macrophages infected with live *M. ulcerans* bacilli producing different types of mycolactone and displaying different degrees of virulence. Our work shows that macrophages infected with highly virulent *M. ulcerans* produce much lower levels of TNF, as compared with macrophages infected with intermediate or nonvirulent strains. The reduced production of TNF, including at low MOIs, was not due to an early death of infected macrophages, since the production of the inflammatory chemokine macrophage inflammatory protein-2 (MIP-2) was not inhibited in macrophages infected with the highly virulent strain. Finally, we showed that TNF induced during *M. ulcerans* infection plays a protective role, since TNF-p55-defficient mice were more susceptible to nonvirulent and intermediate virulent strains of *M. ulcerans* when compared to wild-type mice.

IFN-γ is a key cytokine in the activation of the macrophage's microbicidal mechanisms required for the control of intracellular pathogens. We showed that IFN-γ-deficient mice displayed an increased susceptibility to infection by nonvirulent and intermediate virulent strains of *M. ulcerans*, but not to a highly virulent strain. In vitro, IFN-γ-activated macrophages control the proliferation of the nonvirulent strain as well as the intermediate virulent strain at a low MOI. For the nonvirulent strain, the intramacrophage mechanism of control was nitric oxide (NO) dependent, since with the inhibition of NO production, macrophages were no longer able to control the proliferation of this strain. Contrarily, NO production was not detected in macrophages infected with intermediate and highly virulent strains. However, macrophages infected with the intermediate virulent, as well as with the nonvirulent strain, expressed *LRG-47*, being this indicative of phagosome maturation.

In summary, we have shown that *M. ulcerans*, like the other pathogenic mycobacteria, has a phase of intramacrophage residence and multiplication. Additionally, we demonstrated that *M. ulcerans* induces the production of TNF by infected macrophages and that this cytokine plays a protective role in experimental BU; however, mycolactone, the toxin produced by *M. ulcerans*, inhibits the production of this cytokine. We also demonstrate that IFN-γ is required to

the induction of NO and phagosome maturation which are the macrophage's microbicidal mechanisms required to control intracellularly *M. ulcerans* proliferation.

Taken together, the results here presented are in accordance with the data in the literature showing that resistance to *M. ulcerans* infections are associated with CMI and DTH. The peculiarity of the immunology of *M. ulcerans* infections lies in the association of the mycobacterial nature of this microorganism with its unique capacity to secrete a potent cytotoxic exotoxin which influences CMI.

These findings have potential relevance for the development of a vaccine against BU targeting cellular immune mechanisms associated with the activation of the macrophage effector functions.

RESUMO

A bactéria *Mycobacterium ulcerans* é o agente etiológico da úlcera do Buruli (BU), uma doença tropical necrotizante que afecta a pele, causando deformidades terríveis se não for tratada numa fase precoce. Este agente patogénico foi identificado pela primeira vez por MacCallum *et al.* em 1948 na Austrália; no entanto, até à última década, a BU passou, praticamente, despercebida à comunidade científica.

A BU afecta essencialmente comunidades rurais, pobres, de Africa. Diferentes medidas têm sido sugeridas para controlar a BU, no entanto, associado a esta doença existe um estigma social muito forte, sendo que, frequentemente, a BU é vista como uma maldição e não como uma doença. Assim sendo, o desenvolvimento de uma vacina contra a BU seria a estratégia mais adequada para a controlar a doença. Para tal, é essencial aumentar o reduzido conhecimento existente, no que respeita à interacção hospedeiro/agente infeccioso.

A patologia da BU está intimamente associada com a micolactona, uma exotoxina produzida pelo *M. ulcerans*, sendo este o factor de virulência mais importante do agente da BU. É sabido que a micolactona associada à parede celular, ou livre, que se difunde pelos tecidos, induz apoptose e necrose das células do hospedeiro, levando à formação de úlceras. De facto, a necrose dos tecidos e a localização extracelular do *M. ulcerans* são as características histopatológicas distintivas da BU. A extensão das lesões necróticas desprovidas de células que, possivelmente devido à difusão da toxina, se expandem para além das áreas onde o bacilo se encontra, levou à interpretação que o *M. ulcerans* induzia respostas inflamatórias celulares reduzidas ou ausentes. Adicionalmente, a presença de grumos de bacilos livres na área central, acelular, do foco da infecção, levou à caracterização do *M. ulcerans* como uma micobactéria extracelular, uma excepção entre o grupo das micobactérias patogénicas. No entanto, diversos estudos descreveram a ocorrência de imunidade mediada por células (CMI), assim como de hipersensibilidade do tipo retardado (DTH), em doentes com BU, o que sugere um papel efector do macrófago no controlo desta infecção.

A utilização de um modelo animal de infecção, que possibilite a análise completa da lesão em tempos precoces, é essencial para que seja possível entender a interacção de *M. ulcerans* com as células do sistema imunológico, nomeadamente com os fagócitos. Trabalho prévio do nosso laboratório mostrou que, no modelo murino de infecção da almofada plantar, a

infecção subcutânea com *M. ulcerans* induz uma resposta inflamatória celular com a presença de macrófagos e neutrófilos. Adicionalmente, o nosso grupo mostrou que, mesmo em estadios avançados da lesão, as células inflamatórias se encontravam constantemente presentes na lesão, restritas, no entanto, à periferia do centro necrótico acelular.

A constante disponibilidade de fagócitos para interagir com o *M. ulcerans*, nas zonas de infiltrado à periferia da lesão, direccionou o meu trabalho de doutoramento no sentido de reavaliar a interacção deste agente patogénico com os fagócitos do hospedeiro, quer em lesões humanas, quer em infecções experimentais.

Em infecções experimentais no modelo murino, mostrámos que o *M. ulcerans* era fagocitado no tecido subcutâneo em tempos curtos após a infecção. Mais tardiamente, conforme anteriormente descrito, o *M. ulcerans* encontrava-se essencialmente na área necrótica, acelular da lesão. À periferia desta área central necrótica, e nos locais onde encontrava o infiltrado inflamatório, o *M. ulcerans* era observado essencialmente no interior de macrófagos. A análise histopatológica de cortes seriados de 15 amostras de lesões de BU, contendo infiltrados inflamatórios e colhidas de doentes Africanos, confirmou, em 8 amostras, a localização intracelular do bacilo em fagócitos presentes nas áreas periféricas da lesão. In vitro, a baixas multiplicidades de infecção (MOI), mostrámos que o *M. ulcerans* é eficientemente fagocitado por macrófagos, a níveis similares aos encontrados para o *Mycobacterium tuberculosis* e *Mycobacterium bovis* BCG. No interior dos macrófagos, o *M. ulcerans* foi encontrado rodeado pela membrana do fagossoma. Mostrámos, também, que, quer estirpes virulentas, produtoras de micolactona, quer estirpes mutantes, deficientes em micolactona, crescem dentro de macrófagos, quando baixas MOIs são usadas, prevenindo-se, assim, a citotoxicidade associada à produção de micolactona. Após esta fase inicial de residência e multiplicação intracelular, as estirpes produtoras de micolactona provocam a lise dos macrófagos, tornando-se extracelulares. Estes resultados sugerem que o *M. ulcerans* é um agente patogénico intracelular, tal como as outras micobactérias patogénicas, o que explica a ocorrência de CMI e DTH, descritas em infecções humanas e em infecções experimentais.

Após a demonstração da existência de uma fase intracelular no ciclo de vida de *M. ulcerans*, o trabalho focou-se no papel das citocinas que estão associadas à resistência do hospedeiro a agentes patogénicos intracelulares.

O factor de necrose tumoral (TNF) e interferão-gama (IFN-γ) são duas citocinas com um papel chave na activação dos mecanismos microbicidas do macrófago, necessários para o controlo intracelular das micobactérias. Resultados anteriormente publicados mostram que uma fracção lipídica, contendo micolactona, suprime a produção de TNF por monócitos humanos cultivados in vitro. No entanto, a expressão de TNF foi reportada em lesões humanas da BU. Tendo estes aspectos em consideração, considerou-se pertinente estudar o efeito da infecção, por diferentes estirpes de *M. ulcerans*, na produção desta citocina por macrófagos infectados, assim como a relevância biológica do TNF em infecções por *M. ulcerans*.

O nosso trabalho confirmou resultados previamente publicados, mostrando que a micolactona inibe a produção de TNF, de uma forma dependente da dose, e expandiu essas observações, usando um modelo de macrófagos primários infectados com estirpes de *M. ulcerans* que produzem diferentes tipos de micolactona e que apresentam diferentes níveis de virulência. Verificámos, de facto, que macrófagos infectados com estirpes virulentas de *M. ulcerans* produzem menores quantidades de TNF, comparativamente com macrófagos infectados com estirpes avirulentas ou de virulência intermédia. A reduzida produção de TNF, incluindo a baixas MOIs, não se deveu a uma morte prematura dos macrófagos infectados, uma vez que e a produção de proteína inflamatória de macrófagos-2 (MIP-2) não era inibida pelas estirpes virulentas. Finalmente, mostrámos que o TNF induzido após infecção tem um papel protector, uma vez que ratinhos deficientes no receptor P55 do TNF são mais susceptíveis à infecção por estirpes de *M. ulcerans* avirulentas ou de virulência intermédia, quando comparados com ratinhos selvagem.

O IFN-γ é uma citocina chave na activação dos mecanismos microbicidas do macrófago, necessários para o controlo da proliferação de agentes patogénicos intracelulares. Neste trabalho, mostrámos que ratinhos deficientes na produção de IFN-γ são mais susceptíveis à infecção por estirpes de *M. ulcerans* avirulentas ou de virulência intermédia, o mesmo não se verificando com uma estirpe de elevada virulência. In vitro, verificámos que macrófagos activados com IFN-γ controlam a proliferação de estirpes de *M. ulcerans* avirulentas e, também, de virulência intermédia, quando foi testada uma baixa MOI. Para a estirpe avirulenta, os mecanismos de controlo intramacrofágico mostraram ser dependentes da produção de óxido nítrico (NO), uma vez que, inibindo-se a produção de NO, os macrófagos activados deixam de controlar a sua proliferação. Contrariamente à infecção pela estirpe avirulenta, a produção de NO

não foi detectada para as outras estirpes testadas. No entanto, macrófagos infectados com a estirpe de *M. ulcerans* de virulência intermédia expressavam *LRG-47*, o que sugere a ocorrência de maturação do fagossoma.

Em resumo, mostrámos que o *M. ulcerans*, tal como as outras micobactérias patogénicas, tem uma fase de residência e multiplicação intracelular. Mostrámos, também, que o *M. ulcerans* induz a produção de TNF por macrófagos infectados, tendo esta citocina um papel chave na resposta protectora contra infecções experimentais por *M. ulcerans*; no entanto, a micolactona, a toxina produzida por *M. ulcerans*, inibe a produção desta citocina. Demonstrámos ainda, que o IFN-γ é necessário para a indução da produção de NO e para a maturação do fagosoma, sendo estes os mecanismos microbicidas necessários para controlar a proliferação intramacrofágica de *M. ulcerans.*

No seu conjunto, os resultados aqui apresentados estão em acordo com resultados previamente publicados que mostram que a resistência à infecção por *M. ulcerans* está associada com CMI e DTH. A peculiaridade da resposta imune ao *M. ulcerans* encontra-se na associação entre a natureza micobacteriana deste microorganismo e a sua capacidade de produzir uma exotoxina citotóxica, influenciando assim a resposta imune celular.

Estes resultados têm uma relevância potencial para o desenvolvimento de uma vacina contra a BU, tendo como alvo mecanismos imunes mediados por células, associados com a activação dos mecanismos efectores do macrófago.

TABLE OF CONTENTS

ABBREVIATIONS

AFB	Acid-fast bacilli
Ag85	Antigen 85
BCG	Bacillus Calmette-Guérin
BMDM	Bone marrow-derived macrophages
BU	Buruli ulcer
CD	Cluster of differentiation
CFP-10	Culture filtrate protein – 10
CFU	Colony forming units
CMI	Cell-mediated immunity
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
ESAT-6	Early secretory antigen target – 6
GBUI	Global Buruli ulcer initiative
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IL-	Interleukin-
IS	Insertion sequence
LAM	Lipoarabinomannan
LAMP 1	Lysosomal-associated membrane protein 1
LJ	Löwenstein-Jensen
LPS	Lipopolysaccharide
MCP-	Monocyte chemoattractant protein-
MIP-	Macrophage inflammatory protein-
NADPH	Nicotinamide adenine dinucleotide phosphate
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PPD	Purified protein derivative
RANTES	Regulation on activation normal T cell expressed and secreted

RD1	Region of difference 1
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
Th	T helper
TNF	Tumour necrosis factor
VBNC	Viable but nonculturable
WHO	World health organization
WT	Wild-type

THESIS PLANNING

The present dissertation is organized in 5 different chapters. The chapters concerning the experimental work (chapters 2 to 4) are presented in form of articles. The manuscripts presented in chapter 2 and 3 have been published already in *Infection and Immunity*, while the manuscript presented in chapter 4 is being prepared for publication.

In chapter 1, a general introduction to *M. ulcerans* infection is presented. A review of the literature on Buruli ulcer (BU), including a brief historical overview and global distribution, microbiology, *M. ulcerans* virulence factors, transmission of the disease, clinical presentation and histopathology, immune response against *M. ulcerans* including innate and acquired immune mechanisms, strategies used to control and treat BU and the use of experimental models to study *M. ulcerans* infection are presented.

In chapter 2, the work "Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans*" is presented, describing for the first time the intracellular lifestyle of *M. ulcerans*, based on the study of BU lesion samples and samples collected from experimentally infected mice, as well as from cultured macrophages infected with *M. ulcerans*.

In chapter 3, the work "Mycolactone-mediated inhibition of tumor necrosis factor production by macrophages infected with *Mycobacterium ulcerans* has implications for the control of infection" is presented, showing that macrophages infected with different clinical isolates of *M. ulcerans* produce different amounts of tumor necrosis factor (TNF). Additionally, an inhibition of TNF production by the mycolactone produced by intramacrophage *M. ulcerans* is also demonstrated. Finally, the biological relevance of TNF in vivo is presented and discussed.

In chapter 4, the work "Interferon-gamma plays a protective role in *Mycobacterium ulcerans* infection through the activation of nitric oxide production and *LRG-47* expression" is presented, showing that Interferon-gamma (IFN-γ)-activated macrophages control the proliferation of nonvirulent and intermediate virulent strains of *M. ulcerans* in vitro. In accordance, IFN-γ-deficient mice are more susceptible to infection with those strains of *M. ulcerans*. IFN-γ-activated macrophages infected with nonvirulent, but not intermediate or highly virulent, strains of *M. ulcerans* produce high amounts of nitric oxide. Nonetheless, macrophages

infected with intermediate virulent strains express *LRG-47*, being this associated with phagosome maturation.

The general discussion of the present dissertation, as well as the main conclusions of the experimental work, is presented in chapter 5.

OBJECTIVES

Work from our laboratory in experimental *Myocobacterium ulcerans* infections started with the characterization of the inflammatory response induced during infection with different strains of *M. ulcerans* in the mouse footpad, a model that allows the continuous microbiological and histopathological monitorization of the entire lesion (111). In that work it was shown, at the periphery of the lesion, a constant influx of inflammatory cells with a neutrophilic and mononuclear profile. In that specific area of the lesion, *M. ulcerans* was frequently seen co-localizing with macrophages and neutrophils. These initial observations raised additional questions regarding the intracellular/extracellular lifestyle of *M. ulcerans* in the host which are explored in the present dissertation. Furthermore, the relevance of tumor necrosis factor (TNF) and interferon-gamma (IFN-γ) and of the macrophage antimycobacterial mechanisms in *M. ulcerans* infections needs further investigation.

Therefore, the specific aims of the present dissertation are:

TO CLARIFY THE LIFESTYLE OF *M. ULCERANS* IN THE HOST:

Buruli ulcer (BU) has been described as an infection associated with minimal or absent inflammation, caused by the extracellular multiplication of *M. ulcerans*. However, resistance to infection by this pathogen is associated with cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH) responses, both in humans and in animal models. Therefore, this association does not fit the classical description of the histopathology of BU, and, in fact, suggests the existence of an intracellular growth phase of *M. ulcerans* in the host. The first aim of the present dissertation is to clarify this point.

TO ELUCIDATE THE BIOLOGICAL RELEVANCE OF TNF IN EXPERIMENTAL *M. ULCERANS* INFECTIONS:

TNF is an important cytokine in the activation of macrophage's antimicrobial mechanisms and therefore a key cytokine in the control of intracellular pathogens. Although purified mycolactone had been shown to inhibit TNF production by bacterial lipopolysaccharide (LPS)-activated monocytes, the capacity of mycolactone produced by intracellular bacilli to inhibit TNF production and the biological relevance of this cytokine in *M. ulcerans* in vivo infections were never addressed.

TO ELUCIDATE THE MICROBICIDAL MECHANISMS USED BY ACTIVATED MACROPHAGES TO CONTROL *M. ULCERANS* PROLIFERATION:

It has been suggested that IFN-γ is associated with resistance to infections by *M. ulcerans*. Of the macrophage's microbicidal mechanisms activated by IFN-γ, phagosome maturation and nitric oxide production are known to be very important to control, intracellularly, the proliferation of intracellular parasites. To elucidate the relevance of IFN-γ and the macrophage microbicidal mechanisms activated by IFN-γ, leading to control *M. ulcerans* proliferation, in vivo and in vitro, is another aim of the current dissertation.

CHAPTER 1

INTRODUCTION

1.1. *MYCOBACTERIUM ULCERANS* HUMAN INFECTION (BURULI ULCER)

Mycobacterium ulcerans infection causes a devastating, necrotizing, skin disease, known as Buruli ulcer (BU), which can also affect the bone. The first description of *M. ulcerans* was published in 1948, when MacCallum and colleagues reported 6 cases of an unusual skin infection in six Australian patients, caused by acid-fast bacilli (AFB) that could only be cultivated in Löwenstein-Jensen (LJ) medium when the incubation temperature was set lower than for *Mycobacterium tuberculosis* (90). However, the disease was already known for a long time in Africa, where in 1897 Sir Albert Cook had reported ulcerative lesions, most likely caused by *M. ulcerans*, in Uganda (189).

Since its first description, many reports on BU have been done across several tropical countries.

Prior to the 1980s, reports came mainly from sub-Saharan Africa, including Congo (156), Gabon, Nigeria (113), Cameroon, Ghana (18) and Uganda (173, 174). In fact, the designation "Buruli ulcer" came from the Buruli county, in Uganda, where large numbers of cases were described in the 1960s (174). Since the 1980s, an increase in BU cases was reported in West Africa, including Nigeria, followed by reports from Ghana, Liberia, Gabon, Benin, Ivory Coast, and Burkina Faso (78). New foci were also discovered recently in Togo (103) and Angola (16) and older ones have been rediscovered in Cameroon (110). Other epidemic foci are present in some regions of Australia, Papua New Guinea, Sri Lanka and South America (FIG. 1).

Nowadays, BU is the third most common mycobacterial infection worldwide, exceeded only by tuberculosis and leprosy (103). Although obtaining data on accurate disease burdens is difficult, it is known that in highly endemic areas, the number of BU cases may even exceed those of tuberculosis and leprosy, affecting up to 22% of the community (9). In Benin, for instance, a recent study has reported detection rates of 21.5/100,000 per year, a value higher than for either tuberculosis or leprosy (37). The same was observed in Ghana, where the prevalence in 1999 was estimated to be 20.7/100,000, exceeding that of leprosy (8).

In recent years, in addition to the increase in the actual number of cases in West African countries and Australia, there was also an increasing geographical spread of the disease within these countries (189). Thus far, BU has been reported in at least 30 countries, mainly in tropical and subtropical regions (FIG. 1), and the number of reported cases is growing, indicating that BU is an emerging infectious disease. The World Health Organization (WHO) recognized the

significance of *M. ulcerans* as an emerging pathogen establishing the Global Buruli ulcer Initiative (GBUI) in 1998. The primary objectives of the GBUI are to raise awareness of the disease, to mobilize support for affected countries, to promote and to coordinate research activities and to coordinate the work of nongovernmental organizations and other partners (190).

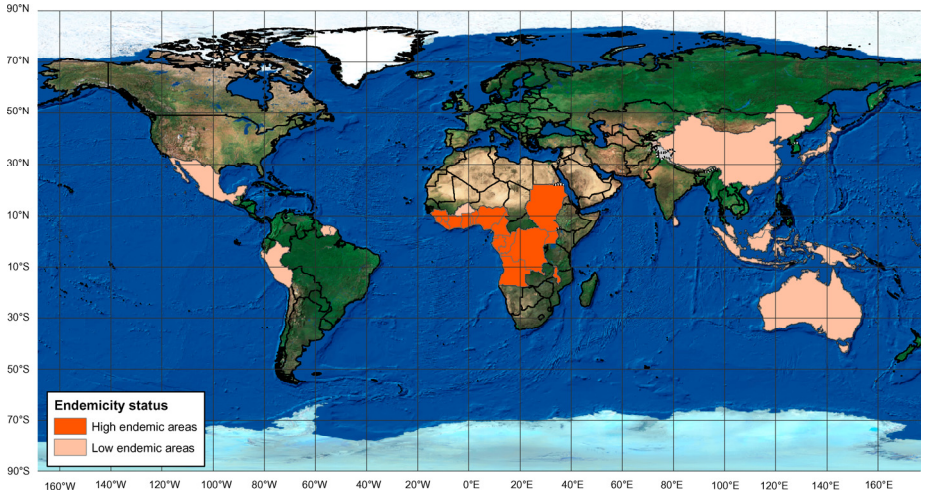

FIG. 1. Countries were Buruli ulcer was reported. Adapted from (78).

More than 50% of those affected with BU are children under the age of 15 years who live in remote rural areas and have little or no access to health services (13). Mortality caused by BU is low, but morbidity is high. In poor rural areas of Africa, a high percentage of BU patients seek medical treatment too late, when extensive lesions, that cause severe disabilities, have already taken place (77). Accordingly, in a recent study in Ghana, of the 75 patients admitted to the study, 45 (58%) had a reduced range of motion of the affected joint (47). In fact, patients try to cure BU within their community by self-treatment with herbs or by visiting local healers (162). One reason for delay in seeking treatment is financial difficulties (162). This is not surprising, since BU results in very high indirect costs for patients and their families (13). However, fear of the treatment, such as amputation, and social stigma are also reasons pointed by patients for not seeking medical care (14, 162).

The disabilities caused by BU have a great impact in the patient's life. Some discontinue school or work due to the social stigma associated with BU (162) or simply because they could not afford school fees after paying the high costs of the treatment (13).

1.2. MICROBIOLOGY OF *M. ULCERANS*

Phylogenetically, *M. ulcerans* is genetically closely related to *Mycobacterium marinum* and *M. tuberculosis* (FIG. 2) (178), two typical intracellular pathogens.

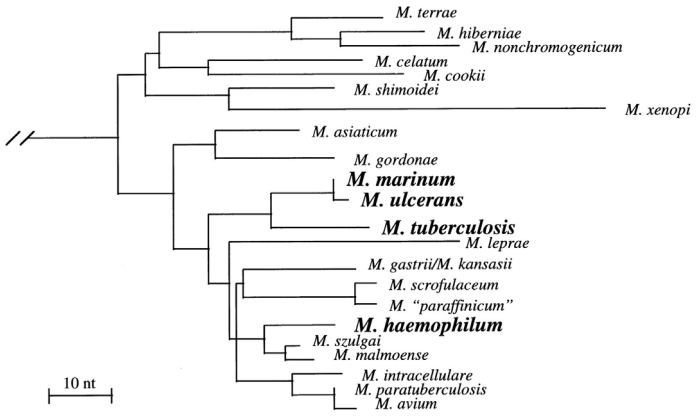

FIG. 2. Phylogenetic tree based on the alignment of partial 16S rRNA gene sequences illustrating the positions of strains of *M. ulcerans*, *M. marinum*, *M. tuberculosis* and *Mycobacterium haemophilum* and other slowly growing mycobacterial species. The tree was rooted by use of *N. asteroides* as an outgroup. The bar indicates a 10-nucleotide (10-nt) difference. Adapted from (178).

Like *M. marinum*, *M. ulcerans* optimal growth temperature is of approximately 32°C and it grows poorly or not at all at temperatures higher than 35°C. Therefore, incubation at 32°C is essential for its isolation in primary culture (126). Attempts to isolate the organism from clinical samples fails in over half of the cases (183). In fact, primary cultures of *M. ulcerans* may take 8 weeks, or more, to become positive (181). While the growth rate is slow, *M. ulcerans* will grow readily on most egg-based media with LJ medium being optimal. It will also grow easily in Middlebrook 7H9 broth media, supplemented with albumin, dextrose and catalase. Another important factor is oxygen concentration. *M. ulcerans* growth was shown to be enhanced at low concentrations of oxygen, suggesting the preference for microaerophilic environments (116).

Since primary cultures of *M. ulcerans* may take several weeks to become positive, and, in some cases, clinically and histopathologically typical Buruli ulcers are reported as culture negative (103, 120, 130), polymerase chain reaction (PCR) amplification of the pathogen's genes is the most widely and accurate method used to detect *M. ulcerans.* Unlike culture, PCR diagnosis permits the confirmation of the clinical diagnosis in a short time.

The first PCR method described for detection of *M. ulcerans* was based on amplification of a region of the 16S rRNA gene (128). The insertion sequences (*IS*)*2404* and *IS2606* have been also used to detect *M. ulcerans* in the environment and in clinical samples (143, 144). However, these insertion sequences were also found in other mycobacteria (193), which indicates that these markers cannot be used as the sole evidence for *M. ulcerans* presence.

1.3. *M. ULCERANS* VIRULENCE FACTORS

A remarkable characteristic of *M. ulcerans* is the production of a unique lipidic family of exotoxins, mycolactones, first isolated and identified in 1999 by George and colleagues (61) (FIG. 3). However, early investigators had already suggested that *M. ulcerans* pathology was closely associated with the production of a toxin (30, 84, 137). The main reason for that suggestion was the fact that BU necrotic lesions extended well beyond the areas where AFB could be seen, and thus only a diffusible toxin could cause the necrosis of the tissue. Recently, the genes required to produce mycolactone were shown to be encoded in a giant plasmid (165).

Mycolactones are hybrid polyketides composed of a lactone core and a fatty acid side chain (69). All strains of *M. ulcerans* recently isolated from BU patients produce a major mycolactone species along with several minor congeners (23, 74, 108), and the pattern of mycolactones produced is conserved in specific geographic areas (108). African strains produce mycolactone A/B as major lipid species, whereas Australian strains produce mainly mycolactone C (108).

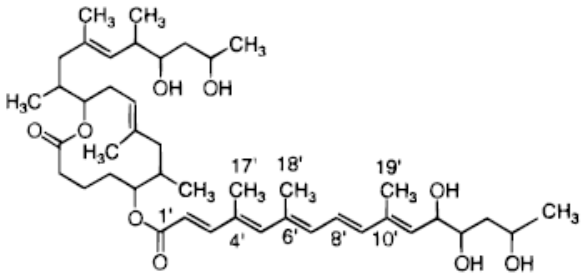

FIG. 3. Mycolactone B structure. Adapted from (69).

In vitro, mycolactone was shown to enter cells through passive diffusion (158), inducing a cell cycle arrest in G_0/G_1 state, followed by apoptosis (62). However, recent in vitro data suggested that apoptosis would only occur in cells when low concentrations of mycolactone are added to the culture medium, the presence of high concentrations of toxin inducing necrosis (4).

However, the possibility has to be considered of mycolactone-induced necrosis representing apoptotic secondary necrosis (111).

Injection of mycolactone into the dermis of guinea pigs is sufficient to induce ulcers (61). On the other hand, mycolactone negative mutant strains of *M. ulcerans* were shown to be nonvirulent for mice and guinea pigs, reinforcing the critical role of mycolactone in *M. ulcerans* pathology (61, 111). In water bugs, unlike wild-type (WT) strains, also mycolactone negative mutant strains failed to colonize the host, suggesting that mycolactone plays a role in the ability of *M. ulcerans* to colonize reservoir species (95).

Although no receptor was identified for mycolactone, a key feature of this toxin is the capacity to inhibit, in vitro, the intracellular signalling of tumour necrosis factor (TNF) (115). In addition, mycolactone was also associated with the reduction of phagocytic uptake of *M. ulcerans* by macrophages (4, 33). Both these effects of mycolactone on macrophages will be later further discussed.

Comparative genomics of mycobacterial strains has revealed several chromosomal regions that are absent from the genomes of the less virulent strains (22, 89, 92). This analysis has focused particular attention on one region, termed region of difference 1 (RD1), which encompasses nine genes (from Rv3871–Rv3879c), because this region is present in virulent *M. tuberculosis* and *Mycobacterium bovis*, but missing in *M. bovis* bacillus Calmette-Guérin (BCG) (89, 92). The function of the RD1 genes still is not known, although two of those genes encode the immunodominant antigens early secretory antigen target – 6 (ESAT-6) and culture filtrate protein – 10 (CFP-10).

An important role for RD1 in mycobacterial pathogenesis has been demonstrated experimentally in several models. Incorporation of *M. tuberculosis* RD1 genes in *Mycobacterium microti* or BCG enhances the virulence of these organisms for mice (134); conversely, a deletion of RD1 genes from *M. tuberculosis* greatly decreases its virulence for mice and cultured macrophages (89). RD1 genes were also shown to be present in some strains of *M. ulcerans* (109). In fact, whereas African, Malaysian, and Australian *M. ulcerans* strains lack RD1 genes, these genes are present in East Asian, Mexican, and South American *M. ulcerans* isolates (109). Studies on the virulence of different *M. ulcerans* isolates in guinea pigs showed that Mexican and Asian isolates were less virulent and induced a greater granulomatous response when compared with African or Australian strains (109). Because ESAT-6 and CFP-10 are strong antigens, the

authors suggested that genes from the RD1 cluster could be involved in the differences in virulence observed (109). However, the highly virulent African strains of *M. ulcerans* lack RD1 while some of the less virulent strains have RD1 (109), which indicates that these genes are not important for *M. ulcerans* virulence.

Recently, phospholipase C and D were identified in whole-cell extracts and culture filtrates of *M. ulcerans* (64). Phospholipases are known to be an important virulence factor for different bacteria (175), including *M. tuberculosis* (54, 82), but in *M. ulcerans* infection the role of these enzymes in the pathogenesis of the disease is still not elucidated.

It is known that noncytotoxic *M. ulcerans* strains do not induce significant pathology in the mouse footpad; however they can persist in mice for at least 12 months postinoculation (111). Therefore, although it is unquestionable that mycolactones play a central role in *M. ulcerans* virulence, more research is required to fully understand the mechanisms of participation of this toxin in the pathogenicity of this mycobacterium as well as to identify other possible virulence factors of *M. ulcerans*.

1.4. TRANSMISSION OF *M. ULCERANS*

There is strong epidemiological evidence that links the source of *M. ulcerans* to swamps and slow-flowing water (17, 185). For instance, the high incidence of Buruli ulcer in the Kinyara refugee settlement, located adjacent to swampy regions near the Nile river, fell substantially when the refugees were moved elsewhere (173). In Nigeria, infections have emerged when a small stream was dammed to make an artificial lake (113). In Phillip Island, Australia, an outbreak of the disease was temporally associated with the formation of a small swamp that, after its improved drainage, was followed by a cessation of cases (185).

Although bodies of stagnant water appear to be the main reservoir of *M. ulcerans* in the environment, Barker and colleagues (17) suggested that contaminated grasses may also provide the means for inoculating *M. ulcerans* in the upper parts of the body (17). Hayman postulated that *M. ulcerans* is an environmental microorganism living in the soil, in symbiosis with the roots of certain plants in tropical rain forests, and that transmission to humans may only occur when the soil microenvironment is disturbed. In those circumstances, *M. ulcerans* may enter in slow moving or stagnant water bodies and start proliferating (70).

Although transmission of BU still is a matter of investigation, most investigators believe that *M. ulcerans* infection is acquired through small injuries in the skin, allowing the entrance of the bacterium to the subcutaneous tissue. Once in the subcutaneous tissue, the low temperature allows *M. ulcerans* growth. Accordingly, Meyers and colleagues showed evidence of antecedent trauma at the site of an *M. ulcerans* lesion (102). This may explain why children under 15 years of age are more affected by BU than adults, and why, among adults, women are more frequently affected (9). In fact, the use of trousers and long-sleeved shirts was reported to be preventive for BU in communities with a high prevalence of the disease (77, 99, 183).

Since, until now, *M. ulcerans* could not be directly cultivated from environmental samples, considering *M. ulcerans* an environmental pathogen is only a hypothesis. *M. ulcerans* genetic material has been detected in the environment by using PCR (143, 164). Although, as discussed above, this technique is not totally safe for the identification of *M. ulcerans* genetic material, the possibility should be considered of *M. ulcerans* existing in the environment in a "viable but nonculturable" (VBNC) state, as has been known to be the case of other pathogens like *Legionella pneumophila* (21). In the VBNC, state the ability to return to an actively metabolizing and culturable form, as well as virulence, is retained (112). It is relevant that mycobacteria are also known to enter the VBNC state (150, 151).

Portaels and colleagues showed the presence of *IS2404* in specimens from water bugs of the Belostomatidae and Naucoridae family and advanced for the first time the hypothesis of BU being transmitted from the environmental niches to humans through insect biting (129). Later, Marsollier and colleagues successfully transmitted the disease to animal models through biting of mice tails by Naucoridae insects, which, after having been fed with *M. ulcerans*-infected grubs (*Phormia terrae novae*), harbored the pathogen in the salivary glands, where they multiply (97). Moreover, *M. ulcerans* was found to colonize the salivary glands of Naucoridae insects in BU endemic areas (97), and the mechanisms through which *M. ulcerans* reaches the salivary glands after being ingested by Naucoridae insects were also recently described (95). In the environment, it is possible that *M. ulcerans* persists in association with aquatic plants that may enhance proliferation and biofilm formation, as it was shown in culture media supplemented with crude extracts from green algae (98). Snails and small fish that feed from these plants may became infected with *M. ulcerans*, as it was experimentally shown (97), concentrating *M. ulcerans* from these sources (46). Higher in the food chain, Naucoridae, or other aquatic predator insects, may became infected after feeding with infected snails or small fish,

concentrating *M. ulcerans* even further and then transmit the disease to humans through biting. In accordance, activities in riverbanks, such as swimming, are considered a risk factor for BU (5). These results support the initial hypothesis (129) that aquatic insects could be vectors of *M. ulcerans* transmission, explaining some of the outbreaks of BU after environmental changes (97). However, the actual relevance of biting by *M. ulcerans*-infected aquatic insects in the transmission of BU is not clarified, and other forms of transmission of *M. ulcerans* to humans, including skin trauma, have also been considered (153).

1.5. CLINICAL PRESENTATION AND HISTOPATHOLOGY OF BURULI ULCER

As discussed before, *M. ulcerans* is genetically very closely related to *M. marinum* and *M. tuberculosis* (FIG. 2). However, and largely due to secretion of mycolactones by *M. ulcerans*, the pathology caused by infection with the agent of BU is unique in key aspects, when compared to other mycobacterioses.

As mentioned above, *M. ulcerans* infection probably starts by the penetration, following a skin trauma (102) or an insect bite (97), of the infectious agent into the subcutaneous tissue where bacilli multiply. The evolution of the disease is dependent on many factors such as the immune status of the host, the size of the inoculum and the characteristics of the *M. ulcerans* strain. The lesions can be nonulcerative (FIG. 4A-D) or ulcerative (FIG. 4E) (http://www.afip.org/Departments/infectious/bu/index.html). If not treated, the initial nonulcerative stages of the disease usually progress to ulcers. In African patients, the disease usually starts as a nodule (FIG. 4A). However, in these patients, pre-ulcerative lesions also include plaques (FIG. 4B), which are large areas of indurated tissue, and oedemas (FIG. 4C) (183). The presentation of pre-ulcerative lesions after infection by *M. ulcerans* appears to vary between strains from different regions. In Australia, the most common form of pre-ulcerative lesions is the papule (FIG. 4D), with no nodular stage (183). It is currently not known why the clinical presentation and the severity of the disease differs among geographical regions, but it might be associated with differences in *M. ulcerans* virulence, including production of different types of mycolactone (108). In accordance, Australian strains produce mycolactone C, a mycolactone known to be less cytotoxic than the mycolactone A/B produced by African strains (108). However, other phenotypic characteristics seem to differentiate African, Australian, and American *M. ulcerans* strains (130).

The active *M. ulcerans* infection develops in association with bacillary multiplication and mycolactone production, with expansion of tissue necrosis and ulcer formation, possibly due to the cytotoxicity of mycolactone and to ischemia associated with vasculopathy (68), which leads to vessel occlusion. Many AFB are present in the infectious focus, but the necrosis extends well beyond bacilli localization (30), possibly due to the diffusion of the *M. ulcerans* toxin, as proposed long ago (30, 84, 137). Ulceration can be extensive and disfiguring, often affecting 50% or more of a limb or extensive areas of the trunk (FIG. 4E). Sometimes, in advanced lesions, the infection spreads, and affects the bone by continuity (contiguous osteomyelitis) or by the haematogeneous route (metastatic osteomyelitis) (121, 127). In Benin, more than 10% of the patients have severe osteomyelitis (37, 87).

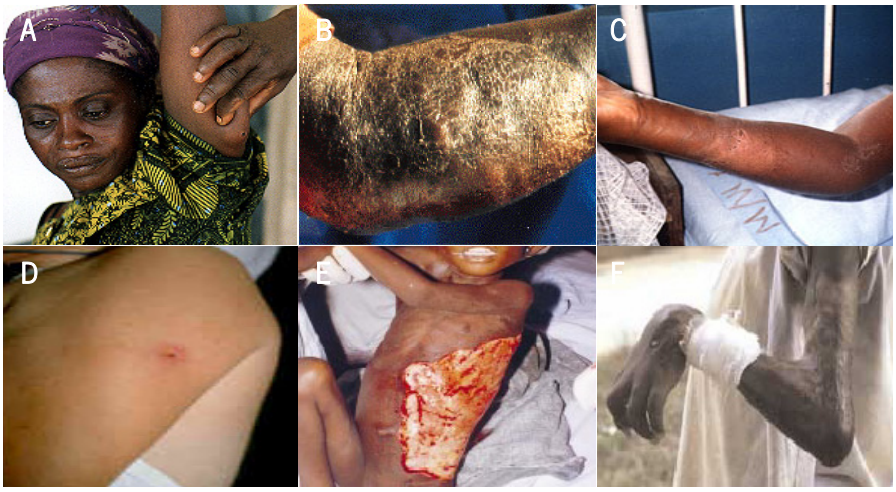

FIG. 4. Clinical forms of BU. A, Nodule; B, papule; C, Oedema; D, Plaque; E, Ulcer; F, Contracture caused by a healed ulcer. Adapted from (189, 190).

Following inoculation of the etiologic agent, the incubation period can vary from weeks to years, but lesions usually take up 2 to 3 months to develop (183). The histopathology of the earliest phases of this process has never been observed in human infections. When available for sudy, infected tissues primarily reveal microcolonies containing large numbers of extracellular AFBs in the central portion of the necrosis. Also common in the necrotic areas are the “ghost cells” (adipocytes that die and lose their nuclei).

The pre-ulcerative stages are symmetrically circumscribed areas of contiguous necrosis in the dermis, with intact epidermis. In the ulcerative stage, a necrotic slough develops at the base of the ulcer and the surrounding skin is undermined, indurated, and hyperpigmentated (1, 183, 189). Ulcerative lesions have been characterized by extensive necrosis of the subcutaneous

tissue and epidermis, alongside clusters of extracellular acid-fast bacilli in the slough. As described below, inflammatory cellular responses have been described as minimal in human samples. Spread of bacilli is known to occur, leading to the development of new lesions, including in the bone, as described above.

Healing of BU lesions is known to occur, characterized by an influx of lymphocytes, macrophages and Langerhans' giant cells. This is followed by the formation of mature tuberculoid granulomas, regeneration and epithelialisation of the epidermis (171, 183). During this healing phase, few if any mycobacteria are found in the sections (30).

1.6. IMMUNE RESPONSE TO INFECTION BY *M. ULCERANS*

1.6.1. INFLAMMATORY RESPONSE TO *M. ULCERANS* INFECTION

Thus far, the immune response against *M. ulcerans* has been far less studied than that against other mycobacteria such as *M. tuberculosis* and *Mycobacterium leprae*. The inflammatory response to mycobacterial infections is characterized histopathologically by an early, acute, predominantly neutrophilic response (117, 146, 149, 154), which changes over time to a chronic, mononuclear, granulomatous pattern (35, 81). This biphasic response is characteristic of infections caused by the *M. ulcerans* related species *M. tuberculosis* and *M. marinum* (25, 81, 105, 117, 149, 179).

In contrast with the above data, *M. ulcerans* infection has been described as inducing low or absent inflammatory responses due to mycolactone production (50). However, the dynamics of the infection process and host inflammatory response at different stages of infection with *M. ulcerans* are still poorly understood, in part due to difficulties in getting samples of human lesions at early stages.

The interpretation that a low or absent inflammatory response is characteristic of BU lesions came from the histopathological observation in BU lesion samples of extensive skin necrotic acellular areas.

Studies on experimental BU are sparse and with contradictory results regarding the inflammatory response induced by *M. ulcerans*. In some studies, the occurrence of an inflammatory response was reported (84, 90, 137) while in others minimal inflammation was

observed (61, 62, 187). For a detailed analysis of the cellular response occurring after infection with different clinical isolates of *M. ulcerans*, including in the initial phases, Oliveira and colleagues, using the mouse footpad model of infection, found that *M. ulcerans* induces relevant, virulence-dependent persistent inflammatory responses (111). A mycolactone-negative, noncytotoxic strain that does not produce progressive infection induces an initial acute neutrophilic response that switches to a chronic mononuclear infiltrate associated with granuloma-like structures, in the absence of necrosis (111). On the other hand, mycolactone-positive, cytotoxic strains multiply progressively and induce a mixed inflammatory response, with neutrophils and some macrophages, that is permanently present during the infectious process, although at spatially restricted areas surrounding a central necrotic, acellular focus that expands with the progress of the disease (111). These results, not only show that *M. ulcerans* induces an inflammatory response, but also that the phagocyte recruitment is biphasic, as it happens with other pathogenic mycobacteria, as reviewed above. In a recent study, Guarner and colleagues reported the occurrence of infiltrates with neutrophils and mononuclear cells in 92% of 78 biopsies of confirmed BU, although such an inflammatory response was recognized as minimal (68).

Neutrophil recruitment to the infection focus is one of the early events after infection. These cells are known to play an important role against extracellular pathogens, but their role in *M. ulcerans* infection is still not known. Nevertheless, several authors had suggested a protective role for this population of cells, early after infection by other pathogenic mycobacteria such as *Mycobacterium avium* or *M. tuberculosis* (117, 119, 139, 154, reviewed by 10). The protective role of neutrophils in these infections would not be through the classical mechanisms of phagocytosis and further destruction of the phagocytosed microorganism, but by transferring neutrophil antimicrobial molecules to macrophages with an increase in their microbicidal activity (154, 167), and by modulating the immune response through the production of Interleukin (IL)-12 and TNF that, in turn, will induce the production of interferon-gamma (IFN-γ) by other cells of the immune system (117, 119).

1.6.2. THE INTRACELLULAR/EXTRACELLULAR LIFESTYLE OF *M. ULCERANS*: A CONTROVERSIAL ISSUE

Present knowledge on BU pathogenesis is mainly based on the analysis of biopsies and surgically excised tissues from human disease, and on the study of animal models of *M. ulcerans* infection. While animal models allow the analysis of the evolution of *M. ulcerans* infections from the beginning, human samples of the initial phases of the infection are not usually available.

Different observations have led investigators to classify *M. ulcerans* as an extracellular pathogen, therefore representing an exception among mycobacteria (32): (i) the presence of large clumps of free bacilli in the centre of the lesion (30), in areas completely devoid of cells; (ii) virulent *M. ulcerans* and mycolactones, under some in vitro conditions, were shown to inhibit the phagocytic activity of macrophages (4, 33); (iii) virulent *M. ulcerans* and mycolactones induce apoptosis of cells in vivo and in vitro (4, 62); and (iv) virulent *M. ulcerans* fails to grow inside macrophages in vitro (136).

Although the histopathological hallmark of BU is the presence of extracellular bacilli in necrotic, acellular areas, because this is the pattern predominating in most biopsies of advanced BU lesions, early investigators have reported *M. ulcerans* bacilli inside macrophages, both in human and in experimental BU. In fact, in the initial description of human *M. ulcerans* infection MacCallum wrote: "The report on the first biopsy specimen drew attention to (...) the distribution of the bacteria in large numbers in phagocytes, recalling that of *Mycobacterium leprae* sometimes seen in skin leprosy" (90). Fenner, by using the experimental footpad infection of mice, described the presence of "scattered mononuclear cells packed with AFB, together with some 'cords' of extracellular acid-fast bacilli" (52). Krieg and colleagues, by using a guinea pig model of infection, described clumps and aggregates of AFB, both within macrophages and free in the tissue (84). Similarly, Read and colleagues found that 24 hours after infection of guinea pigs with *M. ulcerans*, most of the AFB appeared in clumps in polymorphonulear phagocytes and macrophages (137). These authors also showed that, as the infection progresses, many of the phagocytic cells containing bacilli degenerated, leaving clumps of AFB free in the tissue (137). The recent paper by Oliveira and colleagues (111) also described the frequent presence of high number of *M. ulcerans* bacilli colocalizing with inflammatory cells in the mouse footpad model of infection.

On the other hand, resistance to infection by *M. ulcerans* in humans and in animal models has been associated with cell-mediated immunity (CMI) and delayed-type hypersensitivity (DTH) responses (see below) (44, 65, 66, 71, 72, 83, 90, 99, 110, 124, 133, 142, 159, 163, 168, 188).

This set of results does not fit with the current characterization of BU histopathology and with the extracellularity of *M. ulcerans*, and in fact suggests the existence of an intracellular lifestyle of *M. ulcerans* at least during a phase of its residence in the host, as it happens with *M. marinum* or *M. tuberculosis*. However, until now there are no studies reporting intracellular growth of *M. ulcerans*, either in vivo or in vitro. Although it is unquestionable that the presence of necrotic acellular areas with extracellular *M. ulcerans* is a diagnostic hallmark of BU, it is reasonable to admit that such a pattern only represents a partial histopathological counterpart of the host-parasite interactions with relevance for BU pathogenesis. To gather a comprehensive perspective integrating the classical description of BU histopathology, the peculiar features of an infection by a cytotoxic mycobacterium, and the type of immunity associated with the disease, an assessment is required of the dynamics of the interaction *M. ulcerans*/host cells in the entire infectious focus.

1.6.3. ROLE OF MACROPHAGES IN THE IMMUNE RESPONSE TO *M. ULCERANS*

Macrophages are central cells in the innate immune response to *M. tuberculosis* and other pathogenic mycobacteria (2, 145) for several reasons: (i) macrophages express receptors to promote phagocytosis of mycobacteria, being therefore the host cell for these intracellular pathogens and, at the same time, the effector cell of the immune response; (ii) macrophages have several antibacterial mechanisms being able to kill mycobacteria within the phagosome (discussed below); (iii) macrophages are able to present mycobacterial antigens to T cells, therefore being a link between innate and adaptive immune responses; (iv) macrophages produce cytokines and chemokines that recruit and activate new inflammatory cells to the infection focus; and (v) macrophages participate in the formation of the granuloma, an histological structure that is essential to the containment of mycobacterial infections.

Macrophages, being present in all body territories as resident sentinels (184) are likely to be the first cells to encounter and phagocytose invading pathogens, including *M. ulcerans* when

entering the sub-cutaneous tissue. Macrophages initiate the immune response by producing cytokines such as IL-1, IL-6, TNF, IL-12, IL-18 and chemokines such as IL-8 and macrophage inflammatory protein-2 (MIP-2) (the murine homologue of the human IL-8 (170)) (81, 104, 147). The capacity of these macrophages to kill phagocytosed mycobacteria depends on its degree of activation that is modulated by cells recruited to the infection focus. Proinflammatory cytokines, mainly IL-1, IL-6 and TNF, and the chemokine IL-8, produced by infected macrophages not only induce the chemotaxis of other inflammatory cells to the infection focus but also stimulate endothelial cells to produce inflammatory mediators such as regulation on activation normal T cell expressed and secreted (RANTES) protein, MIP-1α and monocyte chemoattractant protein (MCP)-1 and the expression of adhesion molecules that will participate in the recruitment of cells (59, 81, 141).

Some of these cytokines and chemokines have been shown to be expressed in *M. ulcerans* lesions in humans (118, 124, 133), however, not much is known about their biological relevance in the context of BU lesions.

TNF is known to be a critical cytokine in the immune response to mycobacteria. Of the inflammatory cytokines produced by macrophages, TNF is one of the first to be produced upon infection, having an autocrine activity on the macrophage itself, increasing its microbicidal mechanisms (discussed bellow). In the infection focus, this cytokine is likely to be produced not only by macrophages, but also by activated T cells or natural killer (NK) cells (118).

TNF has been shown to be expressed in BU lesions (124, 133), despite the capacity of mycolactone to inhibit its production in vitro (115). In addition, it was recently shown that TNF was expressed in macrophages infected with a mycolactone-negative *M. ulcerans* mutant, but not in macrophages infected with mycolactone-positive *M. ulcerans* (33), suggesting that, in vivo, this cytokine may be produced by newly recruited macrophages but not by infected macrophages.

In addition to its effects on the activation of macrophage's effector mechanisms, TNF is known to have an important role in *M. tuberculosis* granuloma development and maintenance (19, 59). Several authors reported that in BU, granuloma formation is associated with the process of healing of necrotic ulcers (1, 71, 72, 163, 183) and disseminated BU and osteomyelitis may be associated with defects in granuloma formation (73, 163). It is, therefore, possible that in BU, TNF is involved in the granulomatous response associated with the healing phase of the disease, but more studies are required in order to determine the exact role of this cytokine.

A spatial analysis of BU lesions showed expression of IL-8 associated with areas of infiltrating neutrophils (118), suggesting that this chemokine may be important for the recruitment of neutrophils during *M. ulcerans* infection. Similar results were presented by Phillips and colleagues, showing higher expression of this chemokine in *M. ulcerans* infected tissues containing neutrophils (124). In vitro, the mouse homologue of IL-8, MIP-2, was shown to be expressed by macrophages infected with *M. ulcerans* (33). Interestingly, macrophages infected with mycolactone-producing strains were shown to express higher amounts of MIP-2 when compared with macrophages infected with mycolactone negative strains (33). The authors of this report suggested that mycolactone inhibit the production of proinflammatory cytokines but, in turn, stimulate chemokine production (33). The presence of IL-8 in BU lesions do not correlate with the classical description of low or absent inflammation associated with *M. ulcerans* infection, since IL-8 is a powerful neutrophil recruiter (15). A study using the mouse footpad model of *M. ulcerans* infection provided evidence suggesting that the lack of inflammatory infiltrates in the extensive areas of necrosis seen in advanced infections results from the destruction of continuously produced inflammatory infiltrates and not from *M. ulcerans*-induced local or systemic immunosuppression (111).

Other cytokines and chemokines are probably involved in the innate phase of the immune response to *M. ulcerans*. However, until now, there is no solid data on the role of any cytokine or chemokine in the immune response to *M. ulcerans*. A better understanding of the role of cytokines and chemokines in BU will certainly help investigators to develop a specific vaccine against this disease.

After being phagocytosed by macrophages, *M. tuberculosis* and other mycobacteria are retained in the phagosome and can be subjected to intracellular killing via a variety of mechanisms, involving phagosome-lysosome fusion, generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), particularly nitric oxide (NO) (reviewed in 58).

The phagosome is a dynamic structure that interacts with endosomes (early endocytic pathway) and lysosomes (late endocytic pathway), thus forming the phagolysosome. During these interactions vesicle patches of membrane and luminal contents are exchanged, as summarized in the "kiss and run" model (42). Phagocytosed microorganisms will be subjected to intralysosomal degradation by acidic hydrolases, this highly regulated event being one of the most powerful antimicrobial mechanisms of phagocytes (27, 42, 43).

In 1971, D'arcy Hart showed for the first time that *M. tuberculosis* inhibits the fusion of phagosomes with lysosomes (12). This virulence mechanism was later shown to be used by other intracellular parasites (60, 75, 80), and proven to be an important mechanism allowing intracellular parasites to survive in infected macrophages. The inhibition of phagosome-lysosome fusion by *M. tuberculosis* was later suggested to be due to the production of copious amounts of ammonia by the bacilli, which alkalinises the phagosome, preventing its fusion with late endocytic compartments (67). Although it is still not clear what is the mechanism by which ammonia prevents phagosome-lysosome fusion, because other bases capable of raising intralysosomal pH actually promote phagolysosome fusion (67), due to its capacity to produce significant amounts of ammonia, mycobacteria can evade the toxic environment within the lysosome vacuole by diminishing the potency of intralysosomal enzymes via alkalinisation. Later, it was shown that the exclusion of vacuolar ATPase proton pumps from phagosomes containing mycobacteria provides the main mechanism for relative lack of acidification of mycobacterial phagosomes (166).

Although the mechanisms used by mycobacteria to inhibit phagolysosome formation are still not completely understood, it is thought that GTPase Rab family plays an important role. Rabs are small GTPases that regulate membrane fusion in eukaryotic cells. The mechanism by which Rabs control membrane fusion is still not completely understood (41). Of particular interest for mycobacterial phagosomes are Rab5 and Rab7. Rab5 has been strongly implicated in the interactions between phagosomes and the endocytic pathway in macrophages (7, 42, 43, 186). Rab7 regulates late endosomal membrane trafficking (132) and has been implicated in interactions between phagosomes and late endosomal compartments (43). As the phagosome matures, it acquires markers of late endocytic organelles such as Rab7 and lysosomal-associated membrane protein –1 (LAMP-1), a late endosomal/lysosomal marker, and loses markers of early endocytic organelles, such as Rab5 (176).

M. tuberculosis phagosome was shown to retain Rab5 while excluding Rab7, showing the low interaction between phagosome and components of the late endocytic pathway (Reviewed by 41). However, phagosomes harboring live *M. avium* or *M. tuberculosis* acquire LAMP-1 (26, 166, 192), showing that mycobacterial phagosome fuses with vesicles in a dynamic process, excluding others to avoid the harmful effects of acidification.

The production of reactive intermediates is dependent on the degree of activation of the phagocytes. Macrophages, upon activation with appropriate agents, such as IFN-γ and TNF,

produce high amounts of NO, the primary reactive product of nitric oxide synthases (NOS), and related RNI. NO is produced by different isoforms of the NOS enzyme. Although the three forms of NOS are expressed in mammals, NOS2 (also known as iNOS, inducible nitric oxide synthase (191)) is the responsible for the high-output production of NO by macrophages (91). NOS2 protein is only expressed upon activation, being the activation agents dependent on the cell type. In mouse peritoneal macrophages, IFN-γ was the only agent shown to be effective when tested alone (91). On the other hand, bacterial lipopolysaccharide (LPS) alone is enough for the activation of NOS2 in the murine macrophage-like cell line RAW264.7 (91). However, the synergy afforded by the combination of TNF with any of the interferons is particularly important, because ingestion of most microbes elicits autocrine production and action of both TNF and IFN-α/β (91). In this context, another important example of synergy is that between IFN-γ and LPS (191), being LPS a strong inducer of TNF and IFNα/β production by macrophages (91). The physiological relevance of IFN-γ as an inducer of NOS2 is evident in the difficulty with which NOS2 is expressed in the macrophages of mice rendered deficient in IFN-γ (34) or its receptor (76). The biological relevance of NOS2 was already tested for a high number of pathogens by using NOS2 deficient mice or by using nontoxic compounds that inhibit this class of enzymes (91). The high-output NO pathway in macrophages has been shown to be detrimental for the host in some infections, due to the immunosuppressive properties of NO, but in most cases, its inhibition leads to an exacerbation of the infection (91).

The significance of these toxic nitrogen intermediates in the host defence against mycobacterial infections is very well documented in the literature, both in vitro and in vivo, particularly in the murine system (58). In fact, to date, the only mechanism by which macrophages have been shown to kill *M. tuberculosis* is the generation of RNI via the NOS2 enzyme (reviewed in 148). However the sensitivity to RNI by mycobacteria is species-dependent. *M. avium*, for instance, has been shown to be resistant to RNI (11). In fact, NOS2 deficient mice were shown to be more efficient clearing *M. avium* infection when compared with wild-type mice (63).

Although the importance of macrophage's RNI in the host defence against some species of mycobacteria is proven, the role of ROI seems to be not as important. In fact, mycobacteria are capable of evading the toxic effect of ROI by different means (24). For instance, lipoarabinomannan (LAM) in the mycobacterial wall is a potent ROI scavenger. The production of catalase, that converts hydrogen peroxide in water, may also play a role in mycobacterial

virulence (93, 140). Even though, mice deficient in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, responsible for ROI production in the macrophage phagosome, are slightly more susceptible to *M. tuberculosis* infection (3, 31). Therefore, the protective role of ROI in the host defence against *M. tuberculosis* can not be excluded.

Although different, these mechanisms act together, upon macrophage activation, in order to kill phagocytosed mycobacteria. The bacteriostatic activity of nitrites (a moderately stable anion formed by the reaction of NO, in water, with oxygen), is acidic-dependent, reflecting the formation of nitrous acid that dismutes in NO. These findings have impact on the milieu within phagolysosomes of activated macrophages, where low pH could serve to catalyze the recovery of NO and where low pH *per se* may help restrict microbial growth in conjunction with NO or its derivatives (91). Accordingly, *M. tuberculosis* and *M. ulcerans* were shown to be sensitive to acidic $NaNO_2$ (122, 125). NO, by reacting with ROI, might become a more potent product. For example, the product of O_2^- and NO, peroxynitrite ($ONOO^-$) has been shown to have a potent bactericidal activity against *Salmonella enterica typhimurium* (36) and *Escherichia coli* (114).

1.6.4. ADAPTIVE IMMUNE RESPONSES TO *M. ULCERANS*

Pathogenic mycobacteria are the classical example of infectious agents for which the protective response relies on CMI. This is because, as stated before, mycobacteria are found and proliferate inside cells, mainly the macrophage, so that T cell-associated macrophage effector mechanisms rather than antibodies are required to control the infection.

Of the T cells involved in antimycobacterial effector mechanisms, the $CD4^+$ subset is the most important. The important role of this T cell population in the defence against mycobacterial infections is supported by epidemiological evidences, showing the huge number of tuberculosis cases associated with human immunodeficiency virus (HIV) infections. In fact, *M. tuberculosis* is the most common opportunistic agent associated with HIV. Accordingly, HIV^+ purified protein derivative $(PPD)^+$ patients have 8–10% annual risk of developing active tuberculosis, compared to a 10% lifetime risk for PPD^+ HIV^- patients (58). Murine studies have shown, by antibody depletion of $CD4^+$ T cells, adoptive transfer, or the use of gene-disrupted mice, that the $CD4^+$ T cell subset is required for the control of mycobacterial infections (reviewed by 58).

The main effector function of $CD4^+$ T cells in mycobacterial infections is the production of IFN-γ that, in turn, will activate the macrophage functions described above, to control or eliminate the intracellular pathogen. This phenotype of cells is called T helper (Th) 1 and is dependent on IL-12 to develop. Another phenotype of cells, Th2, is dependent on IL-4 to develop and is very important for the control of extracellular parasites, since Th2 cells promote humoral immune responses, with the production of IgG1 and IgE (106).

Unlike tuberculosis or leprosy, only a limited number of studies were reported in animal models of BU designed to study the interaction of *M. ulcerans* with the different cell populations of the immune system. The most relevant data, to be discussed bellow, on the characteristics of the immune response against *M. ulcerans*, have been gathered from BU patients and healthy household contacts of BU patients from Africa, Australia and South America.

As for other mycobacterioses, studies with BU patients suggest that the macrophage-activating cytokine IFN-γ is very important for the control of *M. ulcerans* infection. In fact, peripheral blood mononuclear cells (PBMC) from BU patients, but not from healthy contacts, display a low capacity to produce IFN-γ after in vitro stimulation with *M. ulcerans* bacilli (65, 133). Using PCR, Gooding and colleagues have shown the expression of Th2 cytokines (IL-4, -5, -6) and IL-10 by PMBC from BU patients (66). On the contrary, PBMC from healthy household contacts of BU patients showed a predominant expression of Th1 cytokines, mainly IFN-γ (65, 66). These results were, however, only partially confirmed by Prevot and colleagues (133). These authors could not find Th2 cytokine expression in BU patients, but they did confirm that the expression of IL-10 was higher in BU patients than in healthy household contacts (133). Subsequent analyses of cytokine expression in nodular or ulcerative BU lesions showed a higher expression of IFN-γ and lower expression of IL-10 in the less severe nodular forms in contrast with lower expression of IFN-γ and higher expression of IL-10 in the ulcerative lesions (133). The expression of IL-10 may be associated to the development of severe forms of the disease, since it is well known that IL-10 is a powerful downregulator of IFN-γ production. In fact, it was hypothesized that mycolactone could stimulate the production of IL-10 in the skin (77).

In a more recent study, Phillips and colleagues reported findings that contradict the various observations since they found a lower expression of IFN-γ in BU patients with nodular lesions, although in some cases the production of this cytokine was above the median when compared with the group of ulcerative lesions (123). The suggestion of Prevot and colleagues

(133) that IL-10, and not Th2 cytokines, was responsible for the low IFN-γ responses in BU patients was also not confirmed by Phillips and colleagues (123), since patients with the nodular forms of the disease did show low IFN-γ responses in the presence of higher IL-10 responses, but patients with ulcerative disease had the highest IL-10 responses and, at the same time, showed high IFN-γ expression (123). In fact, these authors did not find any inverse correlation between IFN-γ and IL-10 (123).

Overall, this set of results suggests, but not prove, a protective role for the Th1 type of response in the control of *M. ulcerans* proliferation. This interpretation is in accordance with the reports of patients with progressive noncontrolled lesions being irresponsive to *M. ulcerans* derived antigens (burulin) on skin testing (159). Conversely, when spontaneous healing occurs, the burulin test tends to change from negative to positive (44, 99), indicating that a specific cell-mediated response has developed.

If the control of BU is determined by the ability of the host to mount a protective cell-mediated response, it would be expected that HIV co-infection would influence its course. Some authors suggest that HIV infection may facilitate multifocal aggressive forms of BU (79, 177), but no long-term studies have been published to investigate an association between HIV infection and BU. The studies done so far have reported a low number of co-infections with *M. ulcerans* and HIV, being therefore, insufficient to draw a conclusion (6, 13, 40).

1.7. Control of Buruli ulcer

1.7.1. General Measures

BU has often been referred as a "disease of the poor" affecting, mainly, poor populations in remote rural areas where modern medical facilities are lacking. As other neglected tropical diseases, BU is treatable. However, the treatment can be very expensive, especially in later stages of the disease, with prolonged hospitalization, depending on the severity of the cases and time of presentation (13). Therefore, precautionary measures to prevent new cases of BU and to limit the spread of the disease in poor rural areas, where the cost of the treatment could pose a serious challenge to a struggling economy, are essential.

One of the problems of applying precautionary measures in endemic areas is the fact that the means of transmission of *M. ulcerans* are still not completely known. Nonetheless, the

disease has been associated with water bodies, like slow flowing rivers, ponds, swamps or lakes; one of the precautionary measures that have been recommended is limiting contact of the population with this possible environmental source of *M. ulcerans* (45). However, this could be difficult to implement since these populations depend of the water courses for their daily activities, such as farming. The wearing of trousers and long sleeved shirts when on farms or swamps was shown to be effective to prevent BU (77, 99, 182). However, this is only a partial solution since the infection can affect every part of the body, including the face.

Another important measure is the diagnosis of the disease. In endemic areas, most BU cases are diagnosed on clinical evidence (183) and a number of diseases can be confused with BU disease in each of its clinical stages. The diagnostic tests used to confirm BU are: (i) detection of *M. ulcerans* AFBs in smears stained with the Ziehl-Neelsen technique; (ii) positive culture of *M. ulcerans*; (iii) positive PCR for the detection of *M. ulcerans* DNA; and (iv) histopathological analysis of biopsy specimens. These tests vary in sensitivity, specificity, speed and cost, and in some cases can only be performed in reference laboratories, far from endemic areas (http://www.who.int/buruli/information/antibiotics/en/index1.html). A recently developed dry-reagent-based PCR has been developed that may be used in hospital laboratories in endemic countries (152). Therefore, simple and rapid diagnostic field tests are required in endemic areas to detect and treat BU in early stages.

Education is very important in small rural communities as a precautionary measure (45). People in the community, due to fear of surgery or amputation, seek help from local healers. Education of these communities should focus on detection of early forms of the disease so that they can be treated without extensive surgery. Additionally, patients should be convinced that BU is a result of a mycobacterial infection rather than a result of sorcery (45).

1.7.2. VACCINATION

At the present time, there is no specific vaccine against *M. ulcerans* infection. However, evidence from the literature suggests that *M. bovis* BCG confers some protection against *M. ulcerans*. BCG is a live attenuated strain of *M. bovis* that was derived from a virulent strain of *M. bovis* by continuous in vitro passages, being currently the only vaccine available against tuberculosis.

The first report on the protective effect of BCG against *M. ulcerans* infection was by Fenner in 1957 (53). In an experimental mouse model, the footpad or intravenous BCG administration conferred considerable protection against a small infectious dose, but only a reduced protection against a large dose of *M. ulcerans* given in the contralateral footpad (53).

In the first clinical trial conducted by the Uganda Buruli Group at the Kyniara refugee's settlement, a total of 2075 individuals were included in the study. Of the 1230 subjects with a negative tuberculin skin test, 606 received a BCG vaccination and 624 were left unvaccinated. From May 1967 to September 1968, a total of 79 new cases of BU were detected, 65 of them in the group of negative tuberculin skin test. In this clinical trial, the authors reported a protection rate of 47%. Of the 65 patients with a negative tuberculin skin test to whom BU was diagnosed during the period of the study, 21 had received the BCG vaccine, whereas 44 were unvaccinated (172). However, the protective effect seemed to be short lived, being only detected on the first 6 mounts after vaccination. During the follow up, 73 of the 79 lesions in the population presented as pre-ulcerative lesions. Interestingly, 5 of the 6 ulcerated lesions were in the nonvacinated group and 1 in the BCG-vaccinated group (172), suggesting that BCG could offer some protection against that severe form of BU. However, the number of patients was too low to draw a reliable conclusion. In another study, Amofah and colleagues found that BCG vaccinated BU patients had a shorter duration of the ulcer when compared with non-BCG vaccinated patients (9).

In a different trial in Uganda, BCG vaccination was shown to confer a similar degree of protection as that found in the previous study with an overall protection of 47% (157). Additionally, lesions developing in the vaccinated group, or in those with initial positive tuberculin reactions, were smaller than those in unvaccinated persons. This study also confirmed the short-lived protective effect, since BCG offered no protection after the first year of vaccination (157).

BCG is known to protect against the disseminated extrapulmonary forms of tuberculosis in young children (28, 88, 107, 180), but it is not very effective protecting against pulmonary tuberculosis in adults (28, 29, 55, 56, 160). In BU, BCG seems to confer a higher degree of protection against the severe form osteomyelitis in children (127). In a group of BU patients from Benin, Portaels and colleagues have shown that only 8,7% of children under 15 years old with BCG scar had osteomyelitis, while 25% of children that did not have BCG scar had osteomyelitis. In the adult's group with BCG scar, 15,7% had osteomyelitis, while 35% that did not have BCG scar had osteomyelitis. These results suggest that effective BCG vaccination at birth provides

significant protection against the development of *M. ulcerans* osteomyelitis in children and adults (127).

Although BCG is the most widely used vaccine in the world, the protection it affords against tuberculosis ranges only from 0-80%, depending on the geographical region (28, 29, 55, 56, 160). In recent years, new vaccines have been developed against tuberculosis, with promising results as compared with the classical BCG vaccine. The search for a new vaccine against tuberculosis is centralized in two categories: improving BCG as a vaccine and developing sub-unit vaccines, containing purified immunodominant antigens rather than an entire organism. Improved BCG vaccines have never been tested in *M. ulcerans* infection; however the use of a DNA vaccine encoding the Antigen 85 (Ag85) of *M. bovis* BCG showed to induce strong proliferative Th1 type cytokine responses upon stimulation of cells with BCG or its purified Ag85 complex, in the mouse model (168). Tanghe and colleagues showed also that this vaccine, like BCG, could reduce significantly the bacterial load in the footpads of *M. ulcerans* infected mice (168). However, the kinetics of *M. ulcerans* growth in vaccinated animals would be necessary to disclose if the protective effect is lost over time, like it happens with BCG.

Although *M. ulcerans*, *M. tuberculosis* and *M. bovis* BCG share a large number of highly conserved antigens, even small sequence changes can result in the loss of immunodominant epitopes. Therefore, it was speculated that an attenuated vaccine based on *M. ulcerans* could offer a better and more specific protection than BCG (77). However, this hypothesis remains to be proven.

Being Naucoridae insect bites a possible mode of *M. ulcerans* transmission (97), Marsollier and colleagues (96) hypothesized that Naucoridae salivary proteins, that were shown to cover bacilli transmitted to mice by *M. ulcerans*-infected insects, could be used to develop a vaccine against BU. Indeed, the authors found that mice repeatedly bitten by *M. ulcerans*-free insects became protected and thus, when these mice were exposed to bites by insects harbouring *M. ulcerans,* pre-ulcerative and ulcerative lesions did not develop. Although this protective status did not prevent *M. ulcerans* from proliferating, the bacterial loads were two $\log_{10}$ lower than in control animals. Moreover, these authors showed that the presence of human antibodies against insect salivary proteins was an immune signature correlating with protection in areas in which BU is endemic (96). However, the relevance and contribution of insect biting in

the transmission of *M. ulcerans* still is not known and thus whether or not a vaccine based on insect salivary proteins could be useful against BU awaits further studies (153).

The development of a better or specific vaccine against BU is urgent to prevent a devastating disease with serious complications and high treatment costs that pose a serious challenge to a struggling rural economy and its health system.

1.8. TREATMENT OF BURULI ULCER

Different therapeutic strategies have been attempted to treat BU, including surgical excision of the lesion, antibiotic therapy and hyperbaric oxygenation or elevation of the temperature in the lesion (155). Some of these treatments, although effective to treat BU without surgical excision, that in certain situations can lead to lifetime scars and disabilities, have never been widely used due to the lack of controlled trials, and the high costs.

1.8.1. SURGICAL EXCISION

The treatment of the early phases of the disease by surgical excision of the nodule, under local anaesthesia, is curative (183). Unfortunately, many patients do not present until there is extensive and disfiguring ulceration, when there is no alternative but wide excision followed by skin-grafting, and sometimes even amputation (190).

Surgical excision of the ulcerative lesion is indeed more complicate, especially if extensive contractures, ankylosis caused by ulceration or fibrosis involving joints are seen. In these cases, physiotherapy or plastic surgery may be required. Standard recommendations include that all necrotic tissue is excised, until normal skin and subcutaneous tissue are reached (189). However, relapses are not infrequent. Teelken and colleagues showed that relapse after surgery may occur in 15-47% of cases (169) and thus the WHO now recommends the use of selected antibiotics combined with surgery to prevent relapses of BU (78), as discussed below.

1.8.2. ANTIMICROBIAL CHEMOTHERAPY

Although *M. ulcerans* is sensitive to most antimycobacterial drugs in vitro, including some aminoglycosides, ethambutol, rifampicin, and clarithromycin (131), the use of drugs to treat BU has been disappointing, especially in ulcerative cases of the disease (183). Controlled trials in humans suggest that both clofazamine (138) and cotrimoxazole (51) are ineffective for ulcers and that also rifampin and dapsone combined have limited efficacy for ulcerative lesions (48). Combinations of rifampin with either streptomycin or amikacin, in the mouse footpad model of infection, have shown to be effective, with the size of the mouse footpad decreasing progressively, reduction of the mean colony forming units (CFU) counts and with no relapses (38, 39). A human trial has recently showed that early nodular lesions may be rendered culture negative after a minimum of 4 weeks therapy with rifampicin plus streptomycin (49).

Based on these successful reports and some observational studies, provisional WHO guidelines now recommend the treatment of BU with a combination of rifampicin and streptomycin for eight weeks, as a first-line treatment for all forms of the active disease (http://www.who.int/buruli/information/antibiotics/en/index1.html). With this regimen, it is expected to reduce the indications of surgery or, at least, the extent of surgery and also to decrease the relapse rate (155).

1.8.3. ALTERNATIVE TREATMENTS

Since *M. ulcerans* optimal growth temperature is 32-33°C, it does not affect internal organs and therefore the skin is the affected organ, with the exception of bone involvement in cases of osteomyelitis. Treatments have been tried in several patients by applying heat to the skin lesion, to maintain a local temperature of approximately 40°C. These patients did not require subsequent surgery and all lesions healed without local recurrences (101). In the same way, *M. ulcerans* growth, in vitro, is impaired at high oxygen partial pressures (116). Therefore, hyperbaric oxygenation was suggested as an alternative therapeutic approach, and showed to be effective to treat *M. ulcerans* infection in mice (85, 86).

Although these treatments have been found to be effective in a limited number of patients, and apart from the high cost that may render them impracticable in endemic areas, evidence of efficacy from control trials is lacking (183).

1.9. EXPERIMENTAL MODELS TO STUDY *M. ULCERANS* INFECTION

Experimental models are necessary to study the mechanisms of natural resistance and the formation of acquired protective immunity against mycobacteria, since it is difficult to work with human material, and there are several ethical problems associated with this. *M. ulcerans* is no exception. Moreover, the use of experimental models is essential to understand the initial steps of the immune response against *M. ulcerans*. Indeed, being *M. ulcerans* infections acquired from the environment, and the disease developing for some time without any signs, BU patients usually seek medical advice at later stages of the disease, many times after traditional treatment has failed. Therefore, samples from humans shortly after infection with *M. ulcerans* are usually unavailable and critical aspects of the interaction of *M. ulcerans* with cells of the innate immune system may be lost.

1.9.1. IN VITRO MODELS

As discussed before, *M. ulcerans* infection is probably acquired through a lesion in the skin or a water bug bite. Being macrophages resident cells of different tissues, it is most likely these are the cells that first contact with *M. ulcerans* and start the events of the immune response. Therefore, the study of mycobacteria-macrophage interactions is obviously a priority.

Different studies have been done in vitro to understand the interaction *M. ulcerans*-macrophages. Since it is still not known if *M. ulcerans* is able to grow inside macrophages, as it happens with the related mycobacteria *M. marinum* and *M. tuberculosis*, Rastogi and colleagues used a model of *M. ulcerans* infection in the macrophage cell line J774A.1 to study in vitro the intramacrophagic growth of the bacilli (136). These authors did not find significant growth of *M. ulcerans* when macrophages were infected with the high multiplicity of infection (MOI) of 10:1 (bacilli/macrophage ratio). In a more recent study, Adusumilli and colleagues suggested that both WT and mycolactone-negative mutant *M. ulcerans* strains could survive in J774A.1 macrophages. However, infection with the WT strain at an MOI of 10:1 resulted in destruction of 90% of the monolayer after two days of infection (4).

Although cell lines are a common model to study mycobacteria-macrophage interactions, these cells are genetically altered, and multiply at fast rates, unlike macrophages in vivo. A most widely used model of cell infection is the in vitro culture of bone marrow-derived macrophages (BMDM). These macrophages are derived in vitro from the bone marrow precursors by using L-

cell conditioning medium. This way, it is possible to obtain a pure and homogeneous population of primary macrophages (161). In vitro, these cells maintain their effector functions and respond to IFN-γ activation by producing RNI and ROI. This population of macrophages was suggested to phagocytose *M. ulcerans* in vitro (33). BMDM were also used to study the expression of cytokines after infection with mycolactone-positive or negative strains of *M. ulcerans* (33).

More studies are although necessary to understand the interactions *M. ulcerans*-macrophages: what molecules are required to control *M. ulcerans* proliferation and how IFN-γ that, as discussed above, was suggested to be important for the control of *M. ulcerans,* acts on the macrophage to inhibit *M. ulcerans* proliferation.

1.9.2. IN VIVO MODELS

There is no widely accepted animal model for *M. ulcerans* infection, although many have been used, including mice (52), rabbits (90), guinea pigs (61, 62, 84), calves (135), anole lizards (94), and nine-banded armadillos (187).

The mouse is the most frequently used animal model to study the immune response to mycobacteria, given the huge database of reagents now available, including antibodies to lymphocyte markers, and the growing number of available gene-disrupted strains. Although mice and humans have some known immunological differences (100), this model has been providing very useful information towards a better understanding of the infectious process in other mycobacterial infections, such as tuberculosis (57).

In the same way as *M. leprae*, *M. ulcerans* murine experimental infection is usually carried out in the footpad, given its optimal growth temperature of 32°C. The mouse footpad model of *M. ulcerans* infection was first used by Fenner (52). Since then, this model was shown to be useful to screen the virulence of different *M. ulcerans* isolates, by their capacity to induce footpad swelling (111, 137), and to test in vivo the efficacy of several antimycobacterial drugs against *M. ulcerans* (20, 39). Oliveira and colleagues used the mouse model to evaluate the different stages of the *M. ulcerans* experimental infection and associated inflammatory response (111). As discussed above, the immune response against *M. ulcerans* is poorly known and, due to the high availability of knock-out inbred mouse strains, it will be very important to determine in

this model the biological role of different cell populations, as well as cytokines and chemokines, in the immune response to *M. ulcerans*.

Another animal model widely used for research on *M. ulcerans* experimental infection is the guinea pig. However, few immunological reagents are available for guinea pigs, which limit the ability to perform definitive experimentation. Even though, this model was used to study the inflammatory response after *M. ulcerans* infection. Microscopic changes induced by *M. ulcerans* in guinea pigs were shown to be similar to those of early human infection, proving the usefulness of this animal model (137). The important role of a toxin in the pathogenesis of *M. ulcerans* was shown for the first time in this model (84) before the eventual confirmation by the isolation of purified mycolactone (61).

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CHAPTER 2

Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans.*

Egídio Torrado, Alexandra G. Fraga, António G. Castro, Pieter Stragier, Wayne M. Meyers,
Françoise Portaels, Manuel T. Silva, Jorge Pedrosa.

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Evidence for an Intramacrophage Growth Phase of *Mycobacterium ulcerans*▽

Egídio Torrado,[1] Alexandra G. Fraga,[1] António G. Castro,[1] Pieter Stragier,[2] Wayne M. Meyers,[3] Françoise Portaels,[2] Manuel T. Silva,[4] and Jorge Pedrosa[1]*

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal[1]*; Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*[2]*; Armed Forces Institute of Pathology, Washington, DC*[3]*; and Institute for Molecular and Cell Biology, Porto, Portugal*[4]

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Mycobacterium ulcerans **is the etiologic agent of Buruli ulcer (BU), an emerging tropical skin disease. Virulent *M. ulcerans* secretes mycolactone, a cytotoxic exotoxin with a key pathogenic role. *M. ulcerans* in biopsy specimens has been described as an extracellular bacillus. In vitro assays have suggested a mycolactone-induced inhibition of *M. ulcerans* uptake by macrophages in which its proliferation has not been demonstrated. Therefore, and uniquely for a mycobacterium, *M. ulcerans* has been classified as an extracellular pathogen. In specimens from patients and in mouse footpad lesions, extracellular bacilli were concentrated in central necrotic acellular areas; however, we found bacilli within macrophages in surrounding inflammatory infiltrates. We demonstrated that mycolactone-producing *M. ulcerans* isolates are efficiently phagocytosed by murine macrophages, indicating that the extracellular location of *M. ulcerans* is not a result of inhibition of phagocytosis. Additionally, we found that *M. ulcerans* multiplies inside cultured mouse macrophages when low multiplicities of infection are used to prevent early mycolactone-associated cytotoxicity. Following the proliferation phase within macrophages, *M. ulcerans* induces the lysis of the infected host cells, becoming extracellular. Our data show that *M. ulcerans*, like *M. tuberculosis*, is an intracellular parasite with phases of intramacrophage and extracellular multiplication. The occurrence of an intramacrophage phase is in accordance with the development of cell-mediated and delayed-type hypersensitivity responses in BU patients.**

Mycobacterium ulcerans is the etiologic agent of Buruli ulcer (BU), an emerging, devastating, difficult-to-treat skin disease reported in many countries, mostly in tropical areas (16), and BU has become the third most common mycobacterial infection in humans after tuberculosis and leprosy. BU assumes various nonulcerative clinical forms that can progress to ulcers.

M. ulcerans (26, 41, 47, 59), *M. tuberculosis* (24, 38, 42), *M. marinum* (25, 57), and *M. haemophilum* (23) are known to have cytotoxic activity, *M. ulcerans* being the most cytotoxic of all known mycobacteria. The *M. ulcerans* toxin is a mycolactone, a unique polyketide lipid exotoxin that has a potent destructive activity for cells that provokes the extensive necrotic lesions characteristic of BU (26). Mycolactone is considered to be the major virulence factor of this pathogen (1, 26, 41).

Intracellular parasites are defined on the basis of their lifestyles in the infected hosts and of the types of immune responses elicited (7, 37): they live and multiply predominantly within host cells, typically macrophages, and therefore are able to survive and grow within cultured macrophages. The host immune response against intramacrophage parasites involves mechanisms of cell-mediated immunity (CMI) accompanied by delayed-type hypersensitivity (DTH).

Genetic analysis places *M. ulcerans* very close to *M. marinum* and *M. tuberculosis* (71), two species of mycobacteria that are typical intracellular parasites that grow within macrophages in vivo (7, 12, 15, 31) and in vitro (25, 42, 45, 57, 58, 66) and elicit CMI and DTH responses (7, 9, 12, 14, 15, 18, 46, 72). Several reports indicate that *M. ulcerans* infections are also associated, at least at some stage of the disease, with CMI and DTH (18, 27, 28, 33, 39, 43, 44, 50, 54, 56, 61, 67, 68, 70, 74), strongly suggesting the existence of an intracellular phase in the life cycle of *M. ulcerans* inside macrophages. It is therefore intriguing that *M. ulcerans* has been described as an extracellular pathogen in humans and experimentally infected animals (1, 11, 12, 17, 26, 29, 33, 52), implying that this organism is an exception among pathogenic mycobacteria (12).

The interpretation that *M. ulcerans* is an extracellular pathogen stems mainly from reports that BU tissues show predominantly free bacilli in extensive necrotic acellular areas (8, 17, 20, 29, 35). Other arguments advanced in support of that interpretation are that (i) *M. ulcerans* and mycolactone, under some in vitro conditions, inhibit the phagocytic activity of macrophages (1, 13, 55, 58) and (ii) *M. ulcerans* fails to grow within macrophages in vitro (1, 58).

The observation that extracellular acid-fast bacilli (AFB) predominate in necrotic areas devoid of cells has also led to the concept that the total absence of, or only minimal, inflammation is typical of *M. ulcerans* infections (11, 17, 29, 33, 35). Although such a histopathological pattern is a diagnostic hallmark of BU, it may not reflect the actual host-parasite interactions at the active foci of infection in patients with BU, interactions that so far have not been analyzed in detail. However, a recent publication (32) reported inflammatory infiltrates with neutrophils and mononuclear cells in 92% of 78 BU

* Corresponding author. Mailing address: Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Portugal. Phone: 351-253-604813. Fax: 351-253-604809. E-mail: jpedrosa@ecsaude.uminho.pt.

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specimens with AFB, but no description of the relative locations of bacilli and inflammatory cells was given. Additionally, we have recently described the consistent occurrence of inflammatory infiltrates with neutrophils and macrophages in the mouse model, with *M. ulcerans* bacilli colocalizing with the phagocytic cells and extracellular bacilli in necrotic acellular areas (51). Other, earlier investigators have reported bacilli within macrophages in experimental *M. ulcerans* infections (21, 41, 59). In this context, it is worth recalling that the initial description of BU noted large numbers of bacilli within phagocytes (43).

Because *M. ulcerans* infections are associated with CMI and DTH responses, and because of our previous observations with mice, we postulated that *M. ulcerans* would be an intracellular pathogen with a phase of extracellular existence.

The objective of the present work is to clarify the lifestyle of *M. ulcerans* by reevaluating *M. ulcerans*-phagocyte interactions in BU tissues, mouse tissues, and cultured macrophages. One advantage of the mouse footpad model is that it is possible to systematically observe the entire lesion and surrounding healthy tissues even in advanced infections. We complemented these observations with in vitro studies involving the interactions of *M. ulcerans* with mouse bone marrow-derived macrophages (BMDM) and with the macrophage cell line J774A.1. Our results show that, in a considerable percentage of BU biopsy specimens containing AFB and inflammatory infiltrates, intramacrophage mycobacteria are present in the infiltrates at the peripheries of the necrotic acellular areas. In this model of infection, we also found that virulent mycolactone-producing strains of *M. ulcerans* are efficiently phagocytosed by macrophages in vitro and in vivo, can be found within macrophages during the entire course of infection, and grow intracellularly within cultured macrophages. These data show that *M. ulcerans*, like *M. tuberculosis*, is an intracellular parasite with phases of extracellular and intramacrophage multiplication.

MATERIALS AND METHODS

BU biopsy specimens. We studied 24 paraffin-embedded biopsy specimens from African patients with ulcerative or nonulcerative BU lesions, confirmed by histopathological features and the presence of AFB, from the pathology archives of the Armed Forces Institute of Pathology, Washington, DC. Serial sections of the entire specimens were viewed under the Olympus BX61 light microscope following staining with Ziehl-Neelsen stain (ZN; Merck, Darmstadt, Germany) and hematoxylin (Merck) or methylene blue.

For the isolation of *M. ulcerans* from BU samples, the tissues (±1 g) were homogenized in 1 ml saline, decontaminated, and inoculated onto Löwenstein-Jensen medium. The inoculated tubes were incubated for up to 12 months at 33°C and were observed weekly. For detection of the RD1 gene cluster, primers for *esxA* (Esat-6, 5′-GACAGAACAGCAGTGGAATTTCG-3′ and 5′-CTTCT GCTGCACACCCTGGTA-3′) and *esxB* (Cfp-10, 5′-TTTTGAAGAACGATGC CGCTAC-3′ and 5′-TGACGGATGTTCGTCGAAATC-3′) were used (48). "*M. liflandii*" ITM04-3050 and *M. ulcerans* ITM00-1240 were used as positive and negative controls, respectively.

Mycobacterial strains and preparation of inocula for experimental work. *M. ulcerans* strains were selected based on different degrees of virulence in mice. *M. ulcerans* 98-912 and 97-1116 are highly virulent, and strain 5114 is avirulent (51). The origins and genetic and phenotypic characteristics of strains 98-912 and 5114 have been previously described in detail (51). Strain 5114 contains the RD1 gene cluster (48) and does not produce mycolactones (47, 69). Strain 97-1116 produces mycolactones A and B (47), and we found it to lack the RD1 gene cluster, as is typical of African strains (48). Strain 98-912 possesses the RD1 gene cluster (48) and produces mycolactones A and B slightly different from those in African strains (34). For the determination of the percentage of phagocytosis, *M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur were used as controls. *M. tuberculosis* possesses RD1, and *M. bovis* BCG lacks this gene cluster (6).

The *M. ulcerans* strains were grown on Löwenstein-Jensen medium at 32°C for approximately 2 months, recovered from slants, diluted in phosphate-buffered saline to a final concentration of 1 mg/ml, and vortexed by using 2-mm-diameter glass beads. The number of AFB in the inocula was determined by the method of Shepard and McRae (62) using ZN. The final suspensions revealed more than 90% of the cells to be viable as assessed with the LIVE/DEAD BacLight kit (Molecular Probes, Leiden, The Netherlands).

Animals. Eight-week-old female BALB/c mice were obtained from Charles River (Barcelona, Spain) and were housed under specific-pathogen-free conditions with food and water ad libitum.

Peritoneal model of infection. Mice were infected in the peritoneal cavity with 0.1 ml of *M. ulcerans* suspensions containing 7 $\log_{10}$ AFB of *M. ulcerans* strain 98-912, 97-1116, or 5114. Phosphate-buffered saline was used as a control. At different time points after infection, four mice per group were sacrificed and peritoneal cells were recovered for cytospin preparations by peritoneal lavage. Cytospin preparations were fixed with 10% Formol in ethanol, stained with ZN, and counterstained with Hemacolor (Merck).

Footpad model of infection. Mice were infected in the left hind footpad with 0.03 ml of suspensions containing 6 $\log_{10}$ AFB of *M. ulcerans* 98-912 or 97-1116. The right hind footpad was used as a control.

Culture of murine BMDM and J774A.1 macrophage cell line. BMDM were prepared as previously described (51). For AFB counting and light microscopy analysis, BMDM were seeded in 24-well plates at a density of 5×10^5 cells/well; for electron microscopy, BMDM were seeded in 6-well plates at a density of 3.5×10^6 cells/well. The plates were kept at 37°C in a 5% CO_2 atmosphere. Twelve hours before infection, macrophages were incubated at 32°C in a 5% CO_2 atmosphere and maintained under these conditions thereafter.

Because BMDM survive in vitro for only a short time, the macrophage cell line J774A.1 was used for longer experimental infections. The J774A.1 mouse macrophage cell line was seeded in 12-well plates at a density of 2×10^5 cells/well and incubated at 37°C for 12 h to allow macrophage adherence. Cells were then washed with Dulbecco modified Eagle medium (DMEM) and incubated at 32°C in a 5% CO_2 atmosphere and maintained in these conditions thereafter.

Macrophage infectivity assays. Bacterial suspensions were further diluted in DMEM to obtain the selected multiplicity of infection (MOI; bacterium/macrophage ratio). For the assessment of phagocytosis and of intramacrophage growth of *M. ulcerans*, BMDM and J774A.1 cells were infected with the appropriate number of bacilli and incubated for 4 h at 32°C in a 5% CO_2 atmosphere and then washed with warm Hank's balanced salt solution to remove noninternalized bacteria. For growth experiments, the macrophages were reincubated at 32°C in DMEM for maximum periods of 8 and 15 days, respectively. At 7 and 10 days after infection, J774A.1 cultures were supplemented with fresh medium by adding 1 ml of DMEM per well.

Since the *M. ulcerans* strains used in this study were found to grow in DMEM and to be susceptible to 20 μg/ml of amikacin (data not shown), this concentration of amikacin (Sigma, St. Louis, MO) was added to the culture wells after infection and kept during the whole period of the experiment to prevent extracellular *M. ulcerans* growth (45, 57).

To evaluate the intramacrophage growth of *M. ulcerans*, AFB were counted as follows: macrophages were lysed with 0.1% saponin (final concentration; Sigma), the suspensions were homogenized with 2-mm glass beads, and the AFB were counted (62). Monolayers were not washed previous to AFB counting to avoid losing nonadherent or loosely adherent cells.

Cytological analysis. Adhesion of BMDM was carried out on 13-mm-diameter plastic coverslips (Nunc, Naperville, IL) in 24-well plates. For cytological analysis, plastic coverslips were removed and the cells were fixed with 10% Formol in ethanol, stained with ZN, and counterstained with hematoxylin. Cells were analyzed under a light microscope, and digital images were captured by using an Olympus DP70 camera (Hamburg, Germany).

To quantify dead macrophages, the trypan blue (Gibco, Paisley, United Kingdom) exclusion assay was employed. J774A.1 macrophages were carefully scraped off by using a cell scraper, stained, and counted.

For ultrastructural studies, BMDM cultures were processed for electron microscopy as previously described (63) and viewed and photographed under a Zeiss EM10 electron microscope (Hallbergmoos, Germany).

Histological studies. Human macrophages were labeled with the antibody NCL-LN5 (Novocastra, Newcastle, United Kingdom) that specifically stains the cytoplasm of histocytes and macrophages (5) or with an isotype control antibody. Briefly, the tissue sections were deparaffinized with xylene, followed by sequential immersion in graded ethanol solutions and hydration. The sections were microwave incubated for 15 min with a commercial antigen retrieval solution,

TABLE 1. Clinical data and histopathologic features evaluated for human biopsy specimens from patients with active BU[a]

Origin of specimen	Clinical feature	Extracellular AFB	Inflammation	Intracellular AFB	RD1
Benin	Nonulcerative	+	−	−	ND
Togo	Nonulcerative	+	−	−	Negative
Benin	Ulcer	+	−	−	ND
Democratic Republic of Congo	Ulcer	+	−	−	Negative
Côte d'lvoire	Ulcer	+	+	−	ND
Benin	Nonulcerative	+	+	−	ND
Benin	Nonulcerative	+	+	−	Negative
Côte d'lvoire	Ulcer	+	+	−	ND
Democratic Republic of Congo	Ulcer	+	+	−	ND
Democratic Republic of Congo	Ulcer	+	+	−	ND
Democratic Republic of Congo	Ulcer	+	+	−	ND
Benin	Nonulcerative	+	+	+	ND
Benin	Nonulcerative	+	+	+	ND
Benin	Nonulcerative	+	+	+	Negative
Benin	Nonulcerative	+	+	+	Negative
Democratic Republic of Congo	Ulcer	+	+	+	ND
Democratic Republic of Congo	Ulcer	+	+	+	ND
Benin	Ulcer	+	+	+	ND
Benin	Ulcer	+	+	+	Negative
Total[b]		19 (100)	15 (79)	8 (42)	

[a] +, present; −, absent; ND, not determined.
[b] Number of specimens with the indicated characteristic; numbers in parentheses are percentages of the total number of specimens examined.

citrate buffer heat-induced epitope retrieval (Labvision Corporation, Fremont, CA). Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min, and primary antibody was added and incubated for 1 h. Slides were developed with the Ultravision anti-polyvalent horseradish peroxidase ready-to-use detection system (Labvision Corporation). For diaminobenzidene detection, slides were incubated with the Ultravision large-volume diaminobenzidene substrate system (Labvision Corporation).

Excised tissues of mouse footpads were fixed in 10% phosphate-buffered formalin. Whole paws were decalcified (Thermo Shandon TBD-1; Runcorn, United Kingdom) and embedded in paraffin. Longitudinal sections of the entire paw were stained with ZN. For labeling of mouse macrophages, the specific marker antibody F4/80 or an isotype control antibody was used, according to the procedure referred to above for immunohistochemical staining. All digital images were captured by using an Olympus DP70 camera.

The present study was conducted under the guidelines and approval of the Research Ethics Committee of the Life and Health Sciences Research Institute.

RESULTS

BU biopsy specimens show intramacrophage *M. ulcerans* bacilli in inflammatory infiltrates. *M. ulcerans* has been described as an extracellular parasite, an exception among pathogenic mycobacteria. On the other hand, reports of CMI and DTH responses to *M. ulcerans* infections (18, 27, 28, 33, 39, 43, 44, 50, 54, 56, 61, 67, 68, 70, 74) point to an intracellular residence of *M. ulcerans*. To reevaluate this issue, we started by performing histopathological analyses of BU tissues.

Nineteen specimens from the Armed Forces Institute of Pathology collection from active cases of African BU were selected based on the presence of AFB and the absence of secondary infections. Serial sections were searched for inflammatory infiltrates and for the localization of AFB in the extracellular and intracellular compartments. Among the 19 cases, inflammatory infiltrates were observed in 15 (Table 1). This result agrees with a recent report describing cellular inflammatory responses in a high percentage of BU cases (32). Among the 15 specimens containing inflammatory cells, 8 showed intracellular bacilli (Table 1), as shown for 2 representative specimens (Fig. 1A to F). In addition, NCL-LN5 antibody demonstrated that *M. ulcerans* bacilli were present within macrophages (Fig. 1C). The histopathological hallmark of BU, extracellular bacilli, was found in all cases and was particularly abundant in the necrotic acellular areas (Fig. 1B), while bacilli colocalizing with inflammatory cells were found in the infiltrates at the peripheries of the necrotic areas (Fig. 1A). Some intracellular bacilli appeared as globus-like structures (Fig. 1F) or within phagocytes undergoing apoptosis (Fig. 1E).

Detection of the gene cluster RD1 carried out with *M. ulcerans* strains from six of the studied BU specimens, with and without detectable intracellular bacilli (Table 1), showed that all these strains were RD1 negative as was previously found with other *M. ulcerans* strains isolated from African patients (48). This result suggests that there is no correlation between the occurrence of intracellular bacilli and RD1.

Following experimental infections, *M. ulcerans* bacilli are phagocytosed by resident macrophages in vivo and by BMDM and J774A.1 macrophages in vitro. Murine peritoneal leukocyte populations can be readily and precisely studied, and this model proved useful in the elucidation of host interactions with several species of mycobacteria (3, 53, 65). We have previously reported the recruitment of phagocytes to the peritoneal cavity in response to inoculated *M. ulcerans* (51). Therefore, we used this model to study the early interactions of phagocytes and *M. ulcerans*.

Microscopic analyses revealed that for all the strains of *M. ulcerans* tested (98-912, 97-1116, and 5114), bacilli were phagocytosed by resident macrophages and recruited neutrophils after the first few hours postinfection, as shown in Fig. 2A for strain 98-912. As previously described for experimental peritoneal infections caused by other mycobacteria (65), as well as the livers of mice infected with *Listeria monocytogenes* (30) or

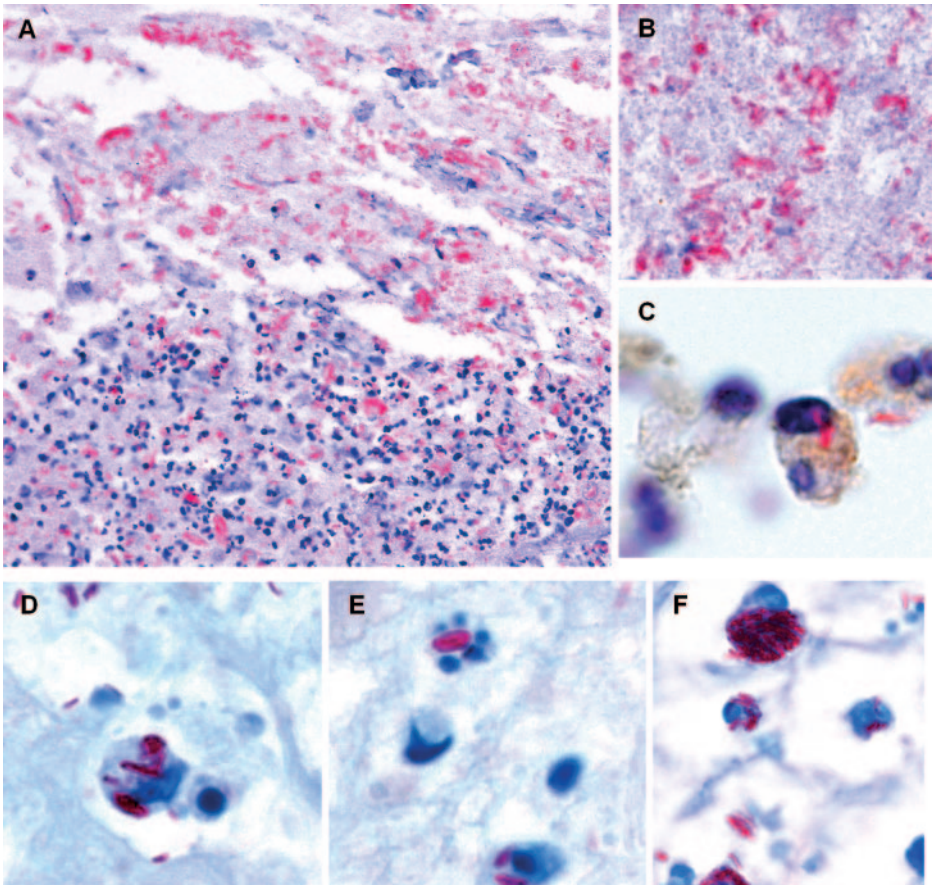

FIG. 1. Intracellular *M. ulcerans* bacilli are found in specific areas of biopsy specimens from BU patients. The two cases shown (A to C and D to F) are representative of the human biopsy specimens showing inflammation and intracellular bacilli. (A) Extracellular bacilli are shown in the necrotic acellular area of the lesion, and bacilli colocalizing with inflammatory cells were found in the peripheral infiltrate. (B) Detail of the abundant extracellular bacilli in the central acellular area. (C) Specifically stained macrophages with internalized bacilli in the peripheral inflammatory area. (D to F) High magnifications of the areas containing inflammatory infiltrates colocalizing with AFB show intracellular bacilli associated with destruction of phagocytes (E) and intracellular, as well as extracellular, globus-like structures (F). Histological sections were stained with ZN and counterstained with hematoxylin or methylene blue. NCL-LN5 antibody was used to specifically label human macrophages. Magnifications, ×250 (A) and ×1,250 (B, C, D, E, and F).

Salmonella enterica serovar Typhimurium (60), we found that neutrophils containing ingested bacilli were progressively phagocytosed by macrophages so that these bacteria were eventually taken up by macrophages.

As in our previous study (51), we consistently found intracellular *M. ulcerans* in the subcutaneous tissue of mouse footpads infected with *M. ulcerans* when the histological analysis of the entire footpad was performed. Mycolactone-producing *M. ulcerans* bacilli (strains 98-912 and 97-1116) were phagocytosed by mononuclear phagocytes and neutrophils a few hours postinoculation (Fig. 2B) and throughout the entire experimental period (Fig. 2B to G). The intramacrophage location of *M. ulcerans* was confirmed by specific immunohistochemical labeling (Fig. 2E). Some infected macrophages contained high numbers of packed clusters of bacilli, reminiscent of globi seen in lepromatous leprosy. These structures were seen both within distended macrophages (Fig. 2G) and free in the extracellular compartment (Fig. 2F and G), probably following the lysis of infected macrophages. Such clusters of AFB in BU have been previously described (10, 43) and were also found by histopathological analysis of BU specimens included in the present study (Fig. 1F). The occurrence of macrophages with high numbers of packed bacilli is suggestive of intracellular multiplication, as in the case of *M. leprae* infections. In the advanced lesions, the central necrotic acellular areas with high numbers of extracellular bacilli were prominent (Fig. 2F and G), but in the boundary between these areas and the surrounding inflammatory infiltrates, clumps of extracellular bacilli were found (Fig. 2F and G). The sizes of the clumps of free bacilli grew as the distance from the limit of the infiltrated areas increased (Fig. 2F and G), suggesting extracellular multiplication of *M. ulcerans*.

We then established an in vitro model of BMDM infection at 32°C to study the interactions of *M. ulcerans* with primary macrophages. Preliminary studies showed that macrophage viability, assessed by cell rounding, shrinkage, and detachment (26), as well as by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling staining (51), was compromised at high MOIs when mycolactone-producing, cytotoxic *M. ulcerans* strains were tested (not shown). An early cytotoxic

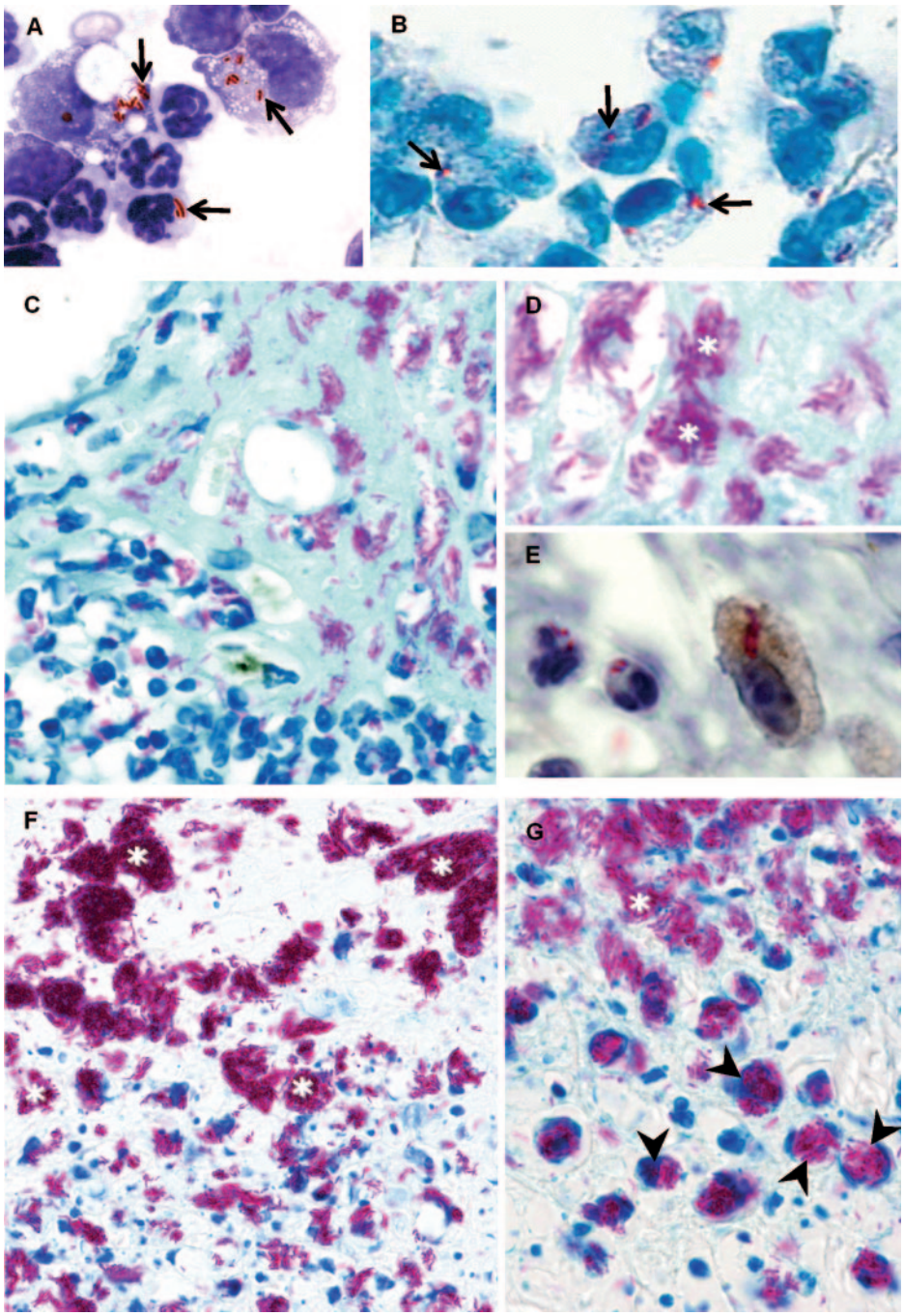

FIG. 2. *M. ulcerans* is phagocytosed by macrophages and neutrophils in vivo following experimental infections. (A) Cytospin preparations of mouse peritoneal leukocytes 6 h after inoculation with 7 $\log_{10}$ AFB of *M. ulcerans* 98-912, stained with ZN and counterstained with Hemacolor. (B to G) Mouse footpads infected subcutaneously with 6 $\log_{10}$ AFB of *M. ulcerans* 98-912, collected 24 h (B) and 15 (C to E) and 28 (F and G) days after infection. (A and B) Bacilli inside neutrophils and macrophages (arrows) early after infection. (C and D) Extracellular bacilli in the necrotic acellular center of the infection focus (shown in high magnification in panel D), surrounded by inflammatory cells with intracellular bacilli. (E) Higher magnification of the peripheral area with inflammatory infiltrates shows specific immunohistochemical staining of a macrophage with internalized bacilli. (F and G) Central acellular necrotic areas and peripheral inflammatory infiltrates in advanced lesions, with both intramacrophage and free bacilli, some packed in globus-like structures with intracellular (arrowheads) or extracellular (white asterisks) locations. Histological sections were stained by ZN and counterstained with methylene blue or hematoxylin. The F4/80 antibody was used to specifically label murine macrophages. Magnifications, ×1,250 (A, B, D, and E), ×550 (C and F), and ×1,000 (G).

effect on macrophages was also described for *M. tuberculosis* and *M. marinum* at high MOIs, being absent at an MOI of 1:1 or lower (25, 42, 57). We therefore tested lower MOIs and found that at an MOI of 1:1 to 1:3, the early cytotoxic effect was abolished. Microscopic analysis showed that *M. ulcerans* 98-912, 97-1116, and 5114 were internalized by macrophages as early as 4 h after infection at a 1:1 MOI, as shown in Fig. 3A for strain 98-912 (see also Fig. 4E for quantitative data). Ul-

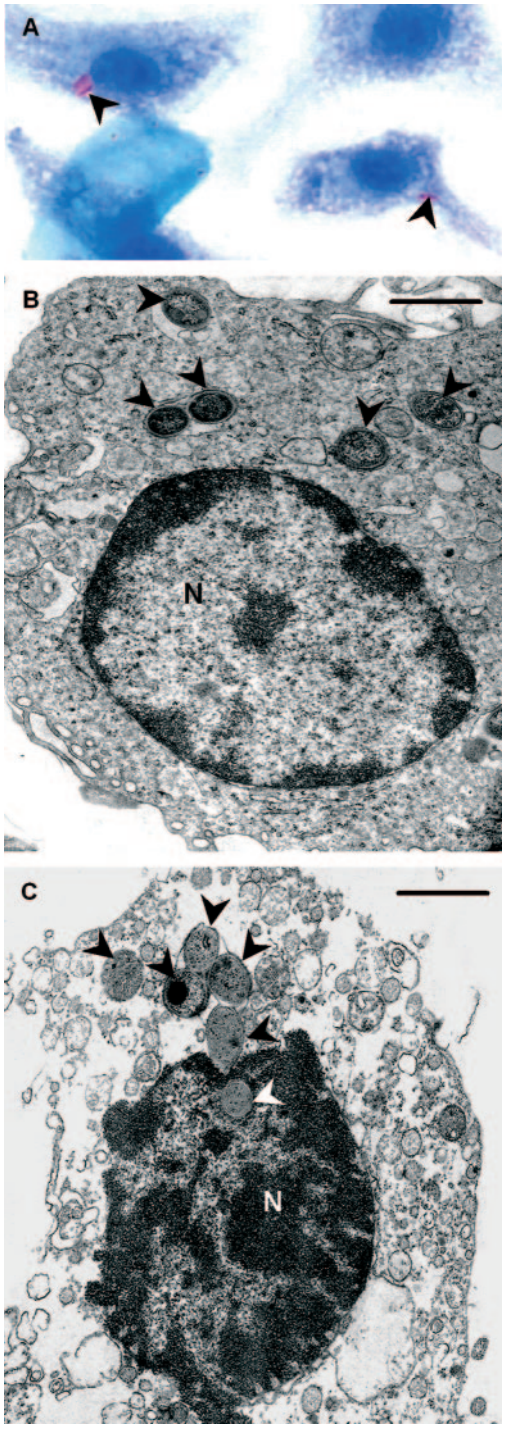

FIG. 3. Intracellular *M. ulcerans* bacilli are found in mouse BMDM infected in vitro. BMDM were infected with *M. ulcerans* 98-912 (MOI, 1:1). (A) Coverslips were removed 4 h postinfection, and the cells were washed, stained with ZN, and counterstained with methylene blue. Microscopic analysis shows bacilli inside macrophages (arrowheads). Magnification, ×1,250. (B) Electron microscopy of BMDM infected for 4 h with *M. ulcerans* 98-912 and photographed 4 days later, showing bacilli inside an intact macrophage (arrowheads). Bar = 1 μm. (C) Six bacilli are seen within a lysing macrophage; one bacillus is inside the nucleus (N), which has ruptured nuclear membranes. This macrophage shows features typical of necrosis (75), namely, rupture of the cytoplasmic and nuclear membranes, condensation of chromatin, swelling and disruption of mitochondria, and loss of the normal density of the cytosol. Bar = 1 μm.

trastructural analysis confirmed the presence of intramacrophage *M. ulcerans*, including the more-cytotoxic strain 98-912 (Fig. 3B and C). Bacilli were present within spacious (Fig. 4A) or tight (Fig. 4B and D) phagosomes, as described previously for other mycobacteria (4). Quantitative assessment of phagocytosis of *M. ulcerans* by BMDM showed that after 4 h at an MOI of 1:1, 26.3% ± 7.5% of the inoculum was internalized for the mycolactone-negative strain 5114; 22.1% ± 4.2% and 29.9% ± 8.7% of the bacteria were within macrophages for the mycolactone-producing strains 98-912 and 97-1116, respectively (Fig. 4E). The phagocytosis index of *M. ulcerans* at an MOI of 1:1 in the macrophage murine cell line J774A.1 was found to be similar to that in BMDM, as shown for strain 97-1116 (Fig. 4E). Similar values were found for *M. tuberculosis* H37Rv and *M. bovis* BCG (30.3% ± 11.4% and 24.9% ± 5.7%, respectively). These results suggest that RD1-positive and RD1-negative mycobacteria behave similarly regarding phagocytosis by BMDM at low MOIs, since *M. tuberculosis* H37Rv and *M. ulcerans* 5114 and 98-912 are RD1 positive (6, 48) and *M. bovis* BCG (6) and *M. ulcerans* 97-1116 (data not shown) are RD1 negative.

Taken together, these results show that soon after experimental infection with *M. ulcerans*, irrespective of the cytotoxicity of the clinical isolate, bacilli are phagocytosed by cultured macrophages as well as by resident macrophages and recruited neutrophils in vivo. Furthermore, intramacrophage bacilli are consistently seen in the inflammatory infiltrates at the peripheries of the necrotic acellular areas where extracellular bacilli accumulate.

***M. ulcerans* proliferates within cultured macrophages.** Following our observation of intramacrophage *M. ulcerans* both in vitro and in vivo, we evaluated the intracellular growth of *M. ulcerans* within BMDM by counting AFB. Amikacin was used to prevent extracellular bacterial multiplication (45, 57). At an MOI of 1:1, we found a 0.6 $\log_{10}$ increase in intracellular AFB counts at 8 days for the mycolactone-negative strain 5114 and the mycolactone-producing strain 97-1116 (Fig. 5A). However, for the most cytotoxic strain, 98-912 (51), bacterial counts did not increase when the same MOI was used (Fig. 5A). This is due to the macrophage damage induced by *M. ulcerans* 98-912, as shown by the necrotic alterations in most infected macrophages (Fig. 3C). Necrotic macrophages lose cytoplasmic membrane integrity (75), permitting access of amikacin to bacilli. However, at an MOI of 1:3, a significant growth of 0.6 $\log_{10}$ was observed over the 8-day experimental period (Fig. 5B), showing that this cytotoxic strain also proliferates inside macrophages when early damage to the macrophages is prevented.

***M. ulcerans* lyses infected macrophages after an initial phase of intracellular proliferation.** We then questioned whether or not the continuous intracellular growth of a cytotoxic *M. ulcerans* strain in macrophages would result in the lysis of the infected cells due to the activity of mycolactone, leading to the shedding of bacilli. To address this question, we infected J774A.1 macrophages, which can be cultured for 15 days, with the mycolactone-producing *M. ulcerans* strain 97-1116 or the mycolactone-negative strain 5114 at different MOIs in the presence of amikacin.

As with infected BMDM, we found that both the 97-1116 and 5114 *M. ulcerans* strains are phagocytosed and grow inside

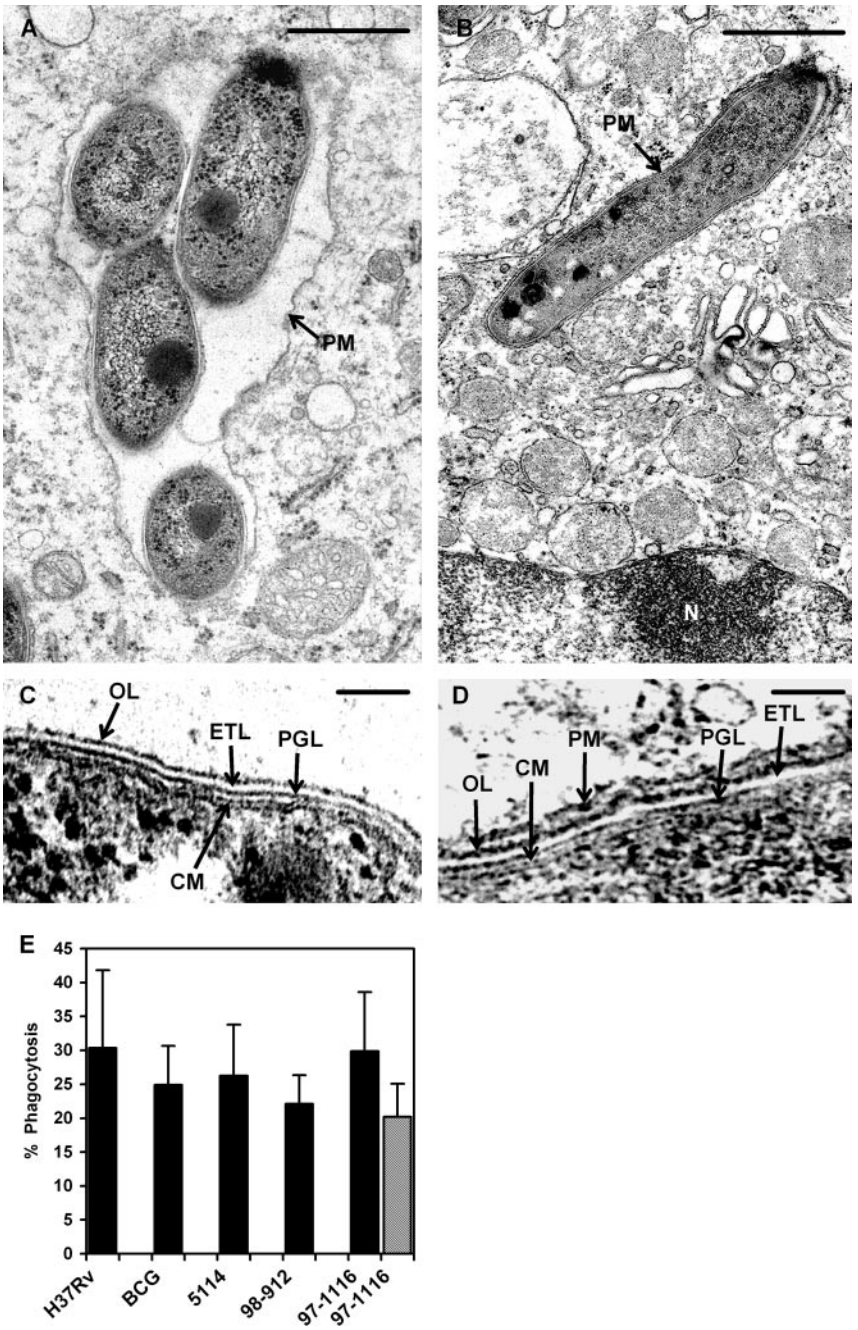

FIG. 4. *M. ulcerans* is found within macrophage phagosomes, and all tested strains are phagocytosed by BMDM at an MOI of 1:1, irrespective of their cytotoxic activity. For electron microscope studies (A to D), BMDM were infected for 4 h with *M. ulcerans* 98-912 (MOI, 1:1) and sampled 4 days after infection. Intracellular bacilli can be found within spacious (A) or tight (B) phagosomes. Bars = 0.5 μm. PM, phagosomal membrane. (C) Detailed image of the cell envelope of extracellular bacilli. The typical layers of mycobacterial envelopes (64) are visible, namely, the cytoplasmic membrane (CM) covered by the cell wall with an innermost electron-dense peptidoglycan layer (PGL), an intermediate electron transparent layer (ETL), and an outer electron-dense layer (OL). Bar = 0.1 μm. (D) High magnification of the bacillary envelope at the zone labeled with an arrow in panel B, showing the same envelope layers shown in panel C plus a tightly apposed phagosomal membrane outside the OL. Bar = 0.1 μm. (E) BMDM (solid bars) were infected for 4 h with *M. ulcerans* 5114, 97-1116, or 98-912 (MOI, 1:1) or, for comparison, with *M. tuberculosis* H37Rv or *M. bovis* BCG (MOI, 1:1). To assess the phagocytosis of *M. ulcerans* by a different macrophagic cell, J774A.1 cultures were infected with *M. ulcerans* 97-1116 as described for BMDM (striped bar). Student's *t* test for the rates of phagocytosis gave nonsignificant *P* values. Results are from one representative experiment out of three independent experiments.

J774A.1 macrophages (Fig. 5C and D). With strain 97-1116 at an MOI of 1:1, the bacterial load increased, reaching 5.68 ± 0.11 $\log_{10}$ AFB by day 10 postinfection, when an arrest in bacterial proliferation was observed (Fig. 5C). At this time point, the number of dead macrophages had increased from 4.35 ± 0.16 $\log_{10}$ at time zero to 5.29 ± 0.15 $\log_{10}$ (an 8.7-fold increase). With strain 5114 at an MOI of 1:1, the bacterial load at 10 days was 5.61 ± 0.10 $\log_{10}$ AFB, with an increase in

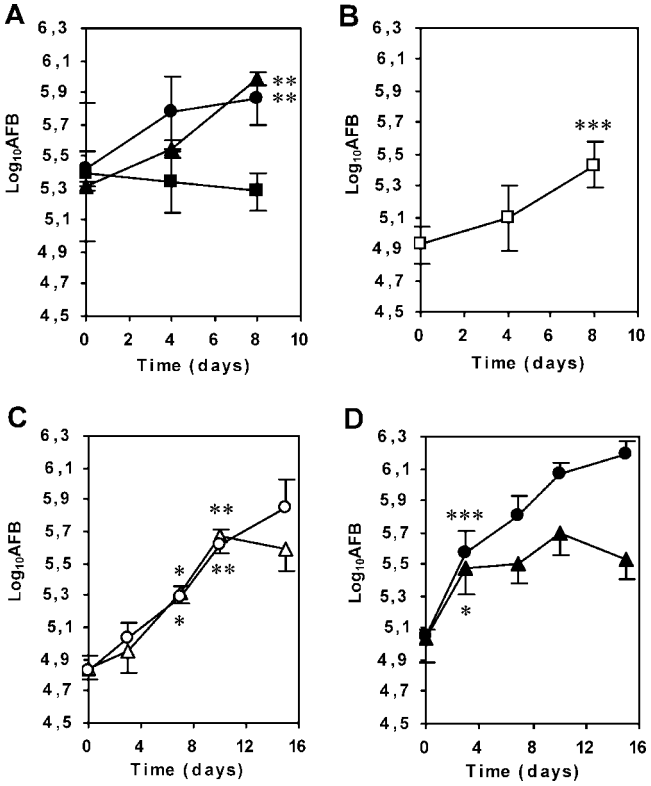

FIG. 5. *M. ulcerans* clinical isolates grow within mouse BMDM and J774A.1 macrophages before inducing cell lysis and becoming extracellular. All macrophage infections were performed in the presence of amikacin. (A and B) BMDM were infected for 4 h at an MOI of 1:1 with *M. ulcerans* 5114 (closed circles), 97-1116 (closed triangles), or 98-912 (closed squares) (A) or at an MOI of 1:3 (open squares) with *M. ulcerans* 98-912 (B). At an MOI of 1:1, *M. ulcerans* 5114 and 97-1116, but not *M. ulcerans* 98-912, proliferate inside macrophages for a period of 8 days. At a lower MOI, the early cytotoxic effect of *M. ulcerans* 98-912 is reduced and intracellular growth was detected. Statistically significant differences between results at day 0 and day 4 or 8, as evaluated by Student's *t* test, are labeled with double asterisks ($P < 0.01$) or triple asterisks ($P < 0.001$). (C and D) J774A.1 cells were infected for 4 h with the mycolactone-positive *M. ulcerans* strain 97-1116 (triangles) or the mycolactone-negative strain 5114 (circles) at an MOI of 1:1 (C) or 3:1 (D) for a period of 15 days. Panels C and D show the numbers of AFB/well, demonstrating that *M. ulcerans* 97-1116 grows inside macrophages before inducing their death and becoming extracellular as indicated by an arrest in bacterial growth (at day 3 for an MOI of 3:1 and day 10 for an MOI of 1:1). In contrast, strain 5114 at both MOIs grew continuously until the end of the experiment. Statistical significance was calculated by using Student's *t* test, comparing the results at each time point with those of the previous time point, and significant differences are labeled with a single asterisk ($P < 0.05$) or double asterisks ($P < 0.01$). Results are from one representative experiment out of two independent experiments.

macrophage mortality of only 1.3-fold; bacterial growth continued, reaching 5.85 ± 0.17 $\log_{10}$ AFB by day 15, when macrophage mortality increased only 1.6-fold. When the higher MOI of 3:1 was tested, the cytotoxic load of strain 97-1116 was achieved at day 3, when the number of AFB reached 5.48 ± 0.17 $\log_{10}$ (Fig. 5D) and the number of dead macrophages was 5.26 ± 0.12 $\log_{10}$ (an 8.1-fold increase). At this MOI, strain 5114 reached 5.58 ± 0.13 $\log_{10}$ AFB at day 3, when macrophage mortality increased only 1.2-fold; growth continued, reaching 6.19 ± 0.09 $\log_{10}$ AFB by day 15. At this time point, the number of dead macrophages had increased only 3.5-fold. In contrast, with strain 97-1116 at the same MOI, the number of dead macrophages had increased 12.6-fold by day 15, with a lower bacterial load (5.54 ± 0.14 $\log_{10}$ AFB).

Altogether, these findings show that after an initial phase of intracellular proliferation, cytotoxic *M. ulcerans* becomes accessible to amikacin, suggesting that lysis of infected macrophages occurs when a certain bacterial load is reached. In vivo, intracellular growth of mycobacteria occurs by cycles of multiplication in individual macrophages followed by their lysis, egress of replicated bacilli, and entry of these bacilli into new macrophages where the growth cycle is repeated (25). The use in our in vitro experiments of amikacin to prevent extracellular proliferation of *M. ulcerans* (45, 57) blocks the spreading of the infection from lysed infected macrophages to healthy macrophages, resulting in an underestimation of the capacity of cytotoxic *M. ulcerans* to grow in cells.

DISCUSSION

The prevailing concept is that *M. ulcerans* is an extracellular pathogen in humans and experimentally infected animals (1, 11, 12, 17, 26, 29, 33, 52). This concept represents an exception among pathogenic mycobacteria (12) and is based mainly on the description of absent or minimal inflammation as a typical feature of *M. ulcerans* infections and on the fact that BU tissues show predominantly free bacilli in extensive necrotic acellular areas (8, 17, 20, 26, 29, 35). In our previous study, we demonstrated with the mouse model of BU that inflammatory infiltrates containing bacilli are consistently present at the peripheries of necrotic acellular areas. From this observation, we concluded that studies directed toward the evaluation of the interactions between phagocytes and *M. ulcerans* in human infections were justified (51). We have shown here that among tissue specimens from 19 BU patients, 8 of 15 specimens with inflammatory exudates and AFB revealed intramacrophage *M. ulcerans* in the inflammatory cells at the peripheries of the extensive necrotic acellular areas. This picture clearly parallels the scenario we described for footpads of mice with experimental BU. Intramacrophage *M. ulcerans* bacilli were readily found in the mouse model, because the entire lesion could be evaluated histopathologically. In specimens from BU patients, however, this phenomenon was not often appreciated because inflammatory exudates and bacilli were not always represented in the tissue fragments. BU lesions are usually extensive, and the chances that a fragment of tissue will fail to include areas of inflammatory infiltrates and bacilli are high. In many instances, intramacrophage *M. ulcerans* bacilli were found only after multiple consecutive sections had been evaluated.

Another argument has been put forward in favor of the concept that *M. ulcerans* is an extracellular pathogen: under some in vitro conditions, *M. ulcerans* and mycolactone inhibit the phagocytic activity of macrophages (1, 13, 55, 58). Extending previous data from our group (51), we now show quantitatively that mycolactone-producing *M. ulcerans* strains are efficiently ingested in vivo as well as by mouse macrophages in vitro at rates comparable to those for a mycolactone-negative *M. ulcerans* strain and for *M. tuberculosis* and *M. bovis* BCG. Our present results suggest that previous data showing inhibi-

tion of phagocytosis of *M. ulcerans* in vitro (1, 55, 58) are explained by the use of high MOIs resulting in early mycolactone-induced damage to the macrophages (26). The only previous study using low MOIs (13) concluded that *M. ulcerans* bacilli "were efficiently captured by RAW264.7 macrophages, bone marrow-derived macrophages, and the dendritic cell line FSDC after a 3 h contact," showing only a partial, dose-dependent inhibition of the phagocytosis of wild-type *M. ulcerans* compared to that of a mycolactone-negative isogenic mutant. These findings and our results indicate that the presence of extracellular *M. ulcerans* in infectious foci is not due to the blockage of phagocytosis and explain the persistent occurrence of intramacrophage *M. ulcerans* in infected tissues. A recent publication on mouse intradermal *M. ulcerans* experimental infection (13) reports intracellular bacilli primarily within neutrophils and rarely in macrophages and only in the initial stages of the infection. Note, however, that a few early studies did report intramacrophage bacilli in experimental (21, 41, 59) and human (43) *M. ulcerans* infections.

The presence of *M. ulcerans* within macrophages in the focus of infection is not evidence that in vivo this mycobacterium is primarily an intracellular parasite. However, using in vitro models of macrophage infection, we show that *M. ulcerans* multiplies intracellularly in macrophages, as is the case for the *M. ulcerans*-related intracellular parasites *M. marinum* and *M. tuberculosis* (25, 42, 45, 57, 58, 66). Previous attempts to grow *M. ulcerans* within mammalian macrophages were unsuccessful (1, 58) due to the cytotoxicity of bacilli for macrophages at the high MOIs used (10:1 to 20:1). We avoided this early cytotoxic effect by using MOIs of 1:1 to 1:3, as previously done with *M. tuberculosis* and *M. marinum* (25, 42, 57). Our data show for the first time that virulent, mycolactone-producing strains of *M. ulcerans* grow inside mammalian macrophages at 32°C.

It would be intuitive to expect intracellular pathogens to have low direct cytotoxicity so that no damage would be imparted to the host cells. Indeed, it has been considered that one of the characteristics of intracellular pathogens is low cytotoxicity (37) and that cytokines produced locally by immune cells would be responsible for mycobacterium-associated cell destruction as seen in pulmonary tuberculosis (14, 22). However, it is becoming progressively evident that intracellular parasites can directly lyse macrophages to exit the cell efficiently after termination of the intracellular proliferation phase and proceed to infect other cells in the same host (extending the infection) or to pass to a new host (spreading the disease) and that direct microbial cytotoxicity is a mechanism that promotes cell lysis. In fact, it is known that many intracellular pathogens, including *M. tuberculosis* and *M. marinum*, have direct cytotoxicity toward professional and occasional phagocytes in vivo and macrophages in vitro (19, 24, 25, 38, 42, 57). In addition, it has been reported that several intracellular parasites, including not only *M. ulcerans*-related mycobacteria (14, 25, 31) but also *Legionella pneumophila*, *Salmonella* spp., and *Shigella* spp. (2, 40, 49, 60), also use cytotoxic mechanisms to lyse infected macrophages at the end of their life cycles within the host cell, thus becoming extracellular.

The consequences of the potentially conflicting features of an intracellular lifestyle and cytotoxicity would be minimized if *M. ulcerans* orchestrated a pattern of transcriptional control of gene expression to turn off the synthesis of mycolactone during intramacrophage growth. The genes involved in mycolactone synthesis would later be switched on, resulting in the lysis of the infected cells and egress of fully replicated bacilli. It has been reported for infections with *Salmonella* spp. (40) and *Legionella pneumophila* (2) that the pathogen machinery responsible for the lysis of host cells is turned off after the entry of the pathogen into the macrophage and expressed only at the end of an intramacrophage multiplication phase. Our results showing that multiplication of *M. ulcerans* at different MOIs within J774A.1 macrophages yields similar maximal bacterial loads and induces similar macrophage viability losses, although at different times, are compatible with the hypothesis of a temporal switching off of mycolactone synthesis within macrophages.

The above-described observations showing that several pathogens are able to reconcile cytotoxicity with intracellular parasitism are in accordance with the concept that low cytotoxicity is a conditional rather than an absolute characteristic of intracellular parasites (37).

In the mouse footpads and in BU tissues from humans, extracellular bacilli colocalized with inflammatory cells but were more abundant in the acellular necrotic areas. The presence of extracellular bacteria is compatible with intracellular parasitism. Accumulation of extracellular bacilli in necrotic areas, following the lysis of macrophages infected with intracellular mycobacteria, has been previously reported for human (31) and murine (36, 73) pulmonary tuberculosis, for human skin lesions due to *M. haemophilum* (17), and for animals infected with *M. marinum* (21, 25).

Our observation of progressive enlargement of the clumps of extracellular AFB away from the areas of colocalization with inflammatory cells suggests that there is extracellular growth of *M. ulcerans* following the shedding of the bacilli from lysed, infected macrophages. Taking into consideration this observation and the results showing that *M. ulcerans* bacilli (i) are efficiently phagocytosed by mouse macrophages in vivo and in vitro, (ii) are present inside macrophages in mouse footpad and peritoneal infections and in BU tissues, and (iii) grow within cultured macrophages, we propose that the growth of *M. ulcerans* would take place, in a manner similar to that of *M. tuberculosis*, by both intracellular and extracellular multiplication in distinct areas of the lesion (14, 31).

According to our model, some of the bacilli shed from lysing macrophages would be taken up by resident macrophages and by monocytes that are continuously attracted to the infectious foci (51) and resume intracellular growth. This continuous intracellular multiplication at the areas of inflammatory infiltrates in active, progressing infections depends on the permanent availability of resident macrophages and incoming monocytes as host cells (51). Our interpretation is that this focus of intramacrophage *M. ulcerans* residence and multiplication at the peripheries of the necrotic areas promotes the progression of the lesion by the invasion of healthy tissues and explains the occurrence of CMI, DTH, and granulomatous responses reported in murine and human *M. ulcerans* infections (18, 27, 28, 33, 39, 43, 44, 50, 54, 56, 61, 67, 68, 70, 74). In contrast, the extracellular bacilli abundant in the central necrotic acellular areas may be ultimately eliminated in the slough of necrotic tissue when the lesion becomes ulcerative, as is the case for

extracellular *M. tuberculosis* in the necrotic caseous foci in human pulmonary tuberculosis (31).

In conclusion, our results showing the uptake of *M. ulcerans* by macrophages in experimentally infected mice and in vitro, the presence of intramacrophage bacilli in inflammatory infiltrates of *M. ulcerans*-infected tissues, and the multiplication of bacilli within cultured macrophages indicate that this mycobacterium fulfils the essential features for classification as an intracellular parasite like the other pathogenic mycobacteria. This interpretation is further supported by the reported protective CMI and DTH responses in mouse and human *M. ulcerans* infections and may have value for the design of novel prophylactic and therapeutic approaches for BU.

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CHAPTER 3

Mycolactone-mediated inhibition of tumor necrosis factor production by macrophages infected with *Mycobacterium ulcerans* has implications for the control of infection.

Egídio Torrado, Sarojini Adusumilli, Alexandra G. Fraga, Pamela L. C. Small, António G. Castro, and Jorge Pedrosa

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Mycolactone-Mediated Inhibition of Tumor Necrosis Factor Production by Macrophages Infected with *Mycobacterium ulcerans* Has Implications for the Control of Infection▽

Egídio Torrado,[1] Sarojini Adusumilli,[2] Alexandra G. Fraga,[1] Pamela L. C. Small,[2] António G. Castro,[1] and Jorge Pedrosa[1]*

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal,[1] and Department of Microbiology, University of Tennessee, M409 Walters Life Sciences, Knoxville, Tennessee 37996-0845[2]

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The pathogenicity of *Mycobacterium ulcerans*, the agent of Buruli ulcer, depends on the cytotoxic exotoxin mycolactone. Little is known about the immune response to this pathogen. Following the demonstration of an intracellular growth phase in the life cycle of *M. ulcerans*, we investigated the production of tumor necrosis factor (TNF) induced by intramacrophage bacilli of diverse toxigenesis/virulence, as well as the biological relevance of TNF during *M. ulcerans* experimental infections. Our data show that murine bone marrow-derived macrophages infected with mycolactone-negative strains of *M. ulcerans* (nonvirulent) produce high amounts of TNF, while macrophages infected with mycolactone-positive strains of intermediate or high virulence produce intermediate or low amounts of TNF, respectively. These results are in accordance with the finding that TNF receptor P55-deficient (TNF-P55 KO) mice are not more susceptible than wild-type mice to infection by the highly virulent strains but are more susceptible to nonvirulent and intermediately virulent strains, demonstrating that TNF is required to control the proliferation of these strains in animals experimentally infected by *M. ulcerans*. We also show that mycolactone produced by intramacrophage *M. ulcerans* bacilli inhibits, in a dose-dependent manner, but does not abrogate, the production of macrophage inflammatory protein 2, which is consistent with the persistent inflammatory responses observed in experimentally infected mice.

Mycobacterium ulcerans is the etiological agent of Buruli ulcer (BU), a devastating, necrotizing skin disease that has increased dramatically over the past decade and has become the third most common mycobacterial infection in humans, after tuberculosis and leprosy (15, 85). In some African tropical areas, the number of BU cases may even exceed those of tuberculosis and leprosy (16, 86). BU has a huge socioeconomic impact in the affected populations and represents an important public health issue in terms of morbidity, treatment, and functional disabilities (3). In addition to the increase in the actual number of cases, there has also been an increasing geographical spread of BU within some tropical countries (86).

Mycobacteria have developed several mechanisms to avoid the harmful effects of the host immune response, including the secretion of soluble factors that have cytotoxic activity against the host cells, the impairment of the bactericidal activity of macrophages, or the modulation of the immune response. *Mycobacterium tuberculosis*, a mycobacterium closely related to *M. ulcerans* (83), secretes culture filtrate protein 10, early secretory antigen target 6, and Man-LAM that exhibit cytotoxic activities against the infected cells or inhibit the production of macrophage-activating cytokines, such as tumor necrosis factor (TNF) (29, 45, 51, 77, 81). Furthermore, it has been suggested that *M. tuberculosis* induces an increased production of interleukin-10 (IL-10), a cytokine with immunosuppressive activity (34). *M. ulcerans* also exhibits cytotoxic activity (32, 50, 56, 67). However, unlike *M. tuberculosis*, *M. ulcerans* cytotoxicity is primarily associated with the production of a unique polyketide lipid exotoxin, mycolactone (32, 33). The genetic basis for mycolactone production was elucidated with the discovery of a giant plasmid that carries all the genes required for mycolactone production (80). It is also known that *M. ulcerans* isolates from different geographical origins produce diverse types of mycolactones (56). The African strains produce mainly mycolactones A/B, whereas Australian strains produce mainly mycolactone C (56). These mycolactones are very similar, with the exception that mycolactone C has one less hydroxyl group on C-12 of the fatty acid side chain (46, 79). Despite this sole difference between mycolactones A/B and C, the latter has been shown to be less cytopathic (46, 56). The different profiles in mycolactone production might contribute to the lower severity of BU disease observed in Australia compared to that in Africa (46). However, it cannot be excluded that production of different amounts of mycolactone may also account for the different degrees of virulence among *M. ulcerans* strains.

In the mouse fibroblast L929 cell line, mycolactone has a cytopathic effect characterized by cell cycle arrest, cell rounding, detachment from the monolayer and, ultimately, apoptosis (31–33, 56). Injection of mycolactone in the guinea pig skin or infection with wild-type (WT) mycolactone-producing *M. ulcerans* induces the formation of ulcers, while infection with a mycolactone-negative mutant strain fails to induce ulceration in this animal model (32). The pathology of *M. ulcerans* infections is therefore closely associated with the secretion of this exotoxin, which is primarily responsible for the extensive ne-

* Corresponding author. Mailing address: Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Portugal. Phone: 351-253-604833. Fax: 351-253-604809. E-mail: jpedrosa@ecsaude.uminho.pt.

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crosis that characterizes BU. Additionally, it has been shown that mycolactone inhibits TNF production (60).

TNF is an effector cytokine produced mainly by macrophages and has an autocrine effect on the activation of the macrophage's microbicidal activity against intracellular parasites, including *Listeria monocytogenes* (39), *M. tuberculosis* (17, 26), *Mycobacterium bovis* BCG (48), and some strains of *Mycobacterium avium* (69). TNF is, therefore, critical for both the antibacterial and the inflammatory host responses against mycobacteria (25). TNF and other cytokines and chemokines produced by macrophages, such as IL-6, IL-1, and macrophage inflammatory protein 2 (MIP-2) (the murine homologue of human IL-8), have an impact on the recruitment of inflammatory cells (5, 18), as well as on the activation and phenotype of T cells (61, 73).

It has been recently shown that *M. ulcerans* has an intramacrophage growth phase (84), which is in accordance with the occurrence of cell-mediated immunity and delayed-type hypersensitivity responses in BU patients (20, 35, 36, 40, 41, 49, 53, 54, 58, 64, 66, 76, 78, 82, 86, 87). The correlation between the amounts of TNF produced by macrophages infected with different strains of *M. ulcerans* and the mycolactones produced by these strains, as well as the biological relevance of TNF in the progression of infection by *M. ulcerans*, is currently unknown. A previous report showed that a lipidic fraction of *M. ulcerans* culture filtrates containing mycolactone suppressed in vitro the production of TNF by human monocytes (60). On the other hand, the expression of TNF is detected in high concentrations in the lesions of BU patients (66). Furthermore, TNF is known to participate in the formation of granulomas (25), which in BU has been shown to occur during the healing phase of the disease (1, 40, 41, 78, 86).

Taking these data into consideration, it is important to study the effects of infection with different strains of *M. ulcerans* on the cytokine production by macrophages, as well as to assess the role of TNF in *M. ulcerans* infection in vivo. A panel of strains of *M. ulcerans* that produce different types of mycolactone or that are mycolactone negative was used in the present work in order to characterize the production of both the proinflammatory cytokine TNF and the chemokine MIP-2 by infected bone marrow-derived macrophages (BMDM). In addition, the biological relevance of TNF production in vivo was assessed by using TNF receptor P55-deficient (TNF-P55 KO) mice infected with different strains of *M. ulcerans*. To further dissect the role of mycolactone on the production of TNF by *M. ulcerans*-infected macrophages, we compared WT *M. ulcerans* with isogenic mutants in which the mycolactone genes were deleted or interrupted by transposon insertion.

We found that BMDM infected with highly virulent but not with intermediately virulent or nonvirulent strains of *M. ulcerans* produced low levels of TNF. In addition, we show that mycolactone inhibits TNF production, in a dose-dependent manner. Interestingly, MIP-2 production was decreased but not abrogated, even in macrophages infected with highly virulent strains. Finally, our data show that TNF induced during *M. ulcerans* infection plays a protective role.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *M. ulcerans* strains used in this study were selected based on their geographical origin and on the type of mycolactone produced (Table 1). Strain 97-1116 is an isolate from Benin that produces a mycolactone A/B that is characteristic of African strains (57). Strain 98-912 was isolated from a Chinese patient and produces a mycolactone A/B slightly different from those in African strains (44). Strain 94-1327 is an isolate from Australia and produces the characteristic mycolactone C (56); 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 (79). Strain 94-1331 is an isolate from Papua New Guinea that is similar to the mycolactone C-producing Australian strains in the sense that its plasmid also lacks the gene MUP053 that is necessary for mycolactone A/B production (79), although it was suggested that this strain can also produce mycolactone A/B (43). Strain 5114 is an isolate from Mexico and does not produce mycolactone (56) due to the loss of key genes involved in the synthesis of this macrolide (79). *Mycobacterium marinum* 00-1026, obtained from a patient living in France, is closely related genetically to *M. ulcerans* and was used as a negative control for mycolactone production (8, 89). Finally, the mycolactone-defective *M. ulcerans* 1615A, a spontaneous mutant, was isolated from nonpigmented colonies of *M. ulcerans* 1615 (32), a strain from Malaysia that produces mycolactone A/B (56). All strains used in this work, except for strain 1615, are from the collection of the Institute of Tropical Medicine, Antwerp, Belgium.

TABLE 1. Mycobacterial strains

Species	Strain	BU clinical form	Geographical origin	Yr of isolation	Type of mycolactone
M. ulcerans	98-912	Ulcer	China	1997	A/B[a]
M. ulcerans	97-1116	Plaque	Benin	1997	A/B
M. ulcerans	1615	Ulcer	Malaysia	1964	A/B
M. ulcerans	94-1331	ND[c]	Papua New Guinea	1994	A/B[b]
M. ulcerans	94-1327	Ulcer	Australia	1994	C
M. ulcerans	5114	Ulcer	Mexico	1953	−
M. marinum	00-1026	−[d]	France	2000	−

[a] Mycolactone A/B slightly different from those in African strains (44).
[b] Like mycolactone C-producing Australian strains, this strain lacks the plasmid gene MUP053 that is involved in mycolactone A/B production (79), although it has been suggested that it also produces mycolactone A/B (43).
[c] ND, not determined.
[d] −, negative result.

The isolates were grown on Löwenstein-Jensen medium at 32°C for approximately 1 month, recovered from slants, diluted in phosphate-buffered saline to a final concentration of 1 mg/ml, and vortexed using 2-mm glass beads. The number of acid-fast bacilli (AFB) in the inocula was determined according to the method described by Shepard and McRae (72), using Ziehl-Neelsen staining (Merck, Darmstadt, Germany). The final suspensions revealed more than 90% viable cells as assessed with a LIVE/DEAD Baclight kit (Molecular Probes, Leiden, The Netherlands).

Animals. Eight-week-old female BALB/c, C57BL/6, and TNF-P55 KO mice were obtained from Charles River (Barcelona, Spain) and were housed under specific-pathogen-free conditions with food and water ad libitum.

Footpad model of infection. Mice were infected in the left hind footpad with 0.03 ml of *M. ulcerans* suspension containing 5 $\log_{10}$ AFB. The right hind footpad was used as a control.

Culture of murine BMDM. Macrophages were derived from the bone marrow as follows: mice were sacrificed with CO_2 and femurs removed under aseptic conditions. Bones were flushed with 5 ml cold Hanks' balanced salt solution (HBSS; Gibco, Paisley, United Kingdom). The resulting cell suspension was centrifuged at 500 × *g* and resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), 10 mM glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Sigma), and 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 × *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 days 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 (59).

Macrophage infectivity assays. 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 in order to obtain the multiplicity of infection (MOI) indicated for each experiment (bacterium/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 for a maximum period of 6 days. To confirm the MOI, counting of AFB in infected macrophages was performed at the beginning of experimental infection as described previously (72).

Mycolactone purification. After purification of mycolactone as described elsewhere (32, 56), the toxin was dissolved in ethanol (100%) and stored at 4°C. The toxin was added to cultured cells by diluting the original preparation in culture medium (DMEM) to a maximum concentration of 0.001% of ethanol. In addition, the wells with control, noninfected macrophages were kept with the same amount of diluted ethanol that was found to be noncytotoxic for macrophages at this residual concentration.

Cytokine analysis by ELISA. At selected time points postinfection, macrophage cultures were centrifuged and the supernatants removed and stored frozen until cytokine analysis by enzyme-linked immunosorbent assay (ELISA). TNF and MIP-2 were measured in the culture supernatant using commercial kits (R&D Systems, Minneapolis, MN) according to the manufacturer's specifications.

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

RESULTS

Different strains of *M. ulcerans* exhibit diverse cytotoxicity in vitro that correlates with mycolactone production and with virulence for mice. It was previously shown that mycolactone A/B-producing *M. ulcerans* bacilli exhibit cytotoxic activity against infected macrophages (59), following an intramacrophage phase of proliferation (84). These same strains induced progressive infections following experimental inoculation of mouse footpads (59). On the other hand, a mycolactone-negative strain was shown to be noncytotoxic and unable to induce lesions in infected footpads (59). In the present work, we expanded our observations by evaluating the cytotoxic activities of a panel of *M. ulcerans* strains that produce diverse types of mycolactone or are nonproducers of mycolactone, using a model of BMDM infection at 32°C. Additionally, we investigated the association between cytotoxicity and virulence for experimentally infected mice.

Macrophages were infected at a 1:1 MOI with the mycolactone-negative strain 5114 or with mycolactone-producing strain 94-1331, 94-1327, 97-1116, or 98-912. As a control, macrophages were infected with *M. marinum* 00-1026, which does not produce mycolactone. The cytotoxicity of *M. ulcerans* was assessed by the occurrence of the typical cytopathic changes induced in cultured cells either by isolated mycolactone (31–33) or by intracellular infection with mycolactone-producing *M. ulcerans* bacilli (59, 84). Those cytopathic changes include cell rounding and detachment from the monolayer followed by cell death (31–33). The evaluation of virulence was performed by measuring over time of the swelling of footpads infected with 5.3 $\log_{10}$ AFB of *M. ulcerans* (59).

Confirming our previous observations (59), we found that the *M. ulcerans* strains 98-912 and 97-1116, which produce mycolactone A/B, were highly cytotoxic, inducing cell rounding, shrinkage, and detachment of more than 90% of cultured macrophages at 6 days postinfection (Fig. 1A), and highly virulent, as shown by the occurrence of footpad swelling between the second and fourth weeks following infection (Fig. 1A). In contrast, no significant alterations were found in monolayers infected with the noncytotoxic, mycolactone-negative strain 5114 or with *M. marinum* 00-1026 (Fig. 1C), which also did not induce measurable footpad swelling up to 25 weeks

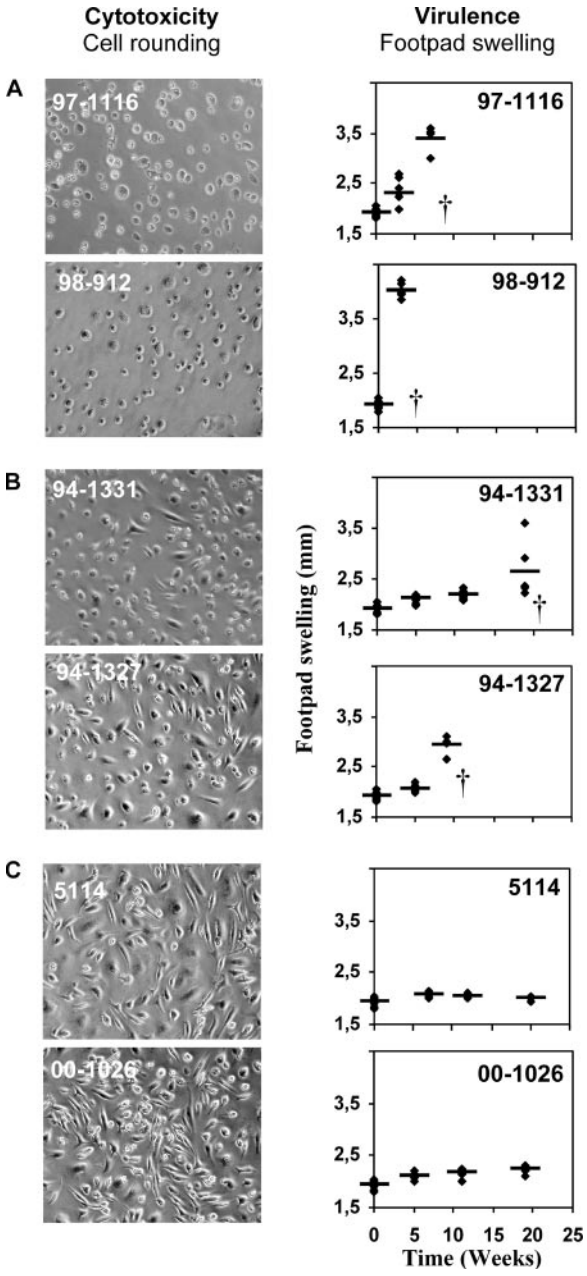

FIG. 1. Cytotoxic activity and virulence of different strains of *M. ulcerans*. (Left) BMDM were infected with different strains of *M. ulcerans* at an MOI (bacilli/macrophages) of 1:1. Macrophages were photographed by phase-contrast microscopy 6 days after infection. *M. ulcerans* cytotoxicity was determined based on the microscopic observation of cell rounding and detachment from the monolayer. (Right) Mice were infected in the left hind footpad with 5.3 $\log_{10}$ AFB of different *M. ulcerans* strains. Virulence was determined by measuring footpad swelling. For ethical reasons mice were sacrificed after the emergence of ulceration. Results are from one representative experiment out of three independent experiments.

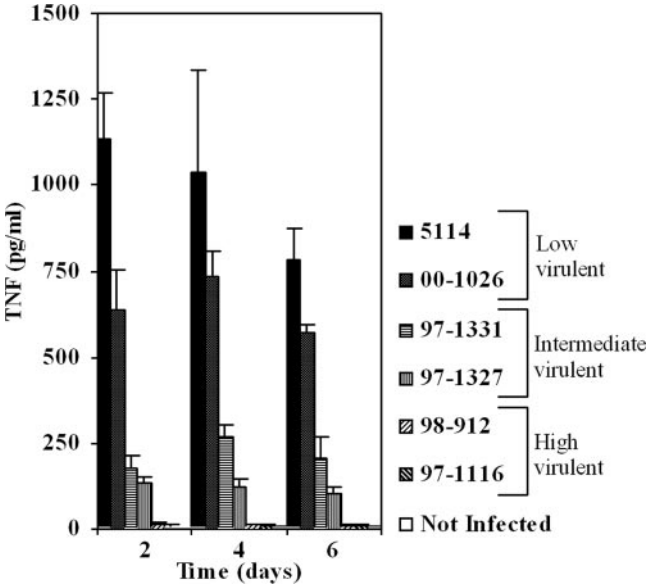

FIG. 2. TNF production by BMDM infected with strains of *M. ulcerans* that produce different amounts and types of mycolactone. Strains of *M. ulcerans* from different origins were used to infect BMDM at an MOI of 1:1. At days 2, 4, and 6 postinfection the supernatants of three independent wells were removed for each strain and frozen until cytokine measurement by ELISA. Low amounts of TNF were produced by macrophages infected with high cytotoxic/virulent strains (98-912 and 97-1116), whereas high amounts of TNF were produced in the case of the low cytotoxicity/low virulence strain 5114 or *M. marinum* 00-1026. Intermediate levels of TNF are produced with strains of intermediate cytotoxicity/virulence (94-1327 and 94-1331). Error bars indicate standard deviations. Results are from one representative experiment out of three independent experiments.

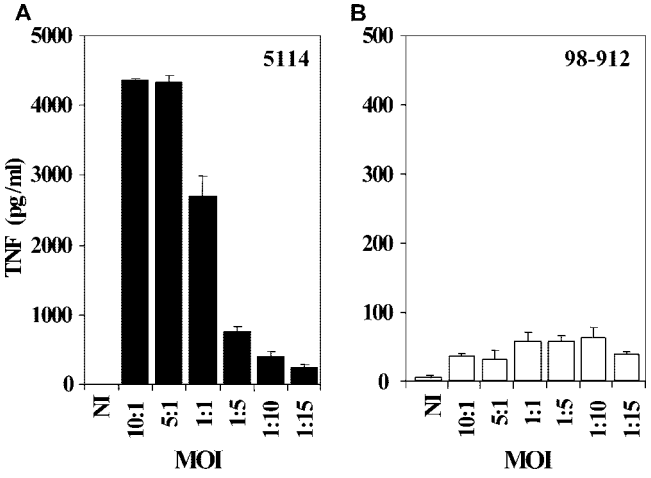

FIG. 3. TNF production by BMDM infected at different MOIs with the mycolactone A/B-producing strain 98-912 or the mycolactone-negative strain 5114. BMDM were infected at MOIs ranging from 10:1 to 1:15 with the *M. ulcerans* strains 5114 (A) or 98-912 (B). Twenty-four hours postinfection, the supernatant from three independent wells was removed for each strain and stored frozen until cytokine measurement by ELISA. BMDM infected with *M. ulcerans* 5114 produce high amounts of TNF at high MOIs, and TNF production decreases as the MOI decreases. With *M. ulcerans* 98-912, production of TNF is low, independent of the MOI. Results are from one representative experiment out of three independent experiments.

postinfection (Fig. 1C). The Australian mycolactone C-producing strain, *M. ulcerans* 94-1327, showed intermediate cytotoxic activity in vitro and intermediate virulence for mice (Fig. 1B). Interestingly, strain 94-1331 from Papua New Guinea, which, like Australian strains, lacks the gene MUP053 that encodes the P450 hydroxylase necessary to hydroxylate C-12 to form mycolactone A/B, also showed an intermediate pattern of in vitro cytotoxicity and virulence (Fig. 1B).

These results confirm the importance of mycolactone as the key pathogenic factor of *M. ulcerans*, showing a correlation between mycolactone production and cytotoxic activity for infected cells and virulence for the host.

BMDM infected with strains of *M. ulcerans* of diverse cytotoxicity/virulence produce different amounts of TNF. Macrophages are key elements in the immune response against intracellular parasites. It is known that among other factors, the autocrine activity of TNF on infected macrophages is critical to control the proliferation of the intracellular mycobacterium *M. tuberculosis* (17, 26) and *M. avium* (69). It was recently shown that *M. ulcerans* has a phase of intramacrophage residence and proliferation before it induces the mycolactone-dependent lysis of infected host cells (84). It is therefore important to study the production of TNF by macrophages infected with different clinical isolates of *M. ulcerans*.

Following the characterization of cytotoxicity/virulence of the panel of *M. ulcerans* strains, we found that infection of BMDM at a 1:1 MOI with those strains led to the production of different amounts of TNF. TNF production was highest in BMDM infected with the mycolactone-negative, nonvirulent strain 5114 or *M. marinum* 00-1026. In cells infected with highly cytotoxic/virulent strains that produce mycolactone type A/B, TNF levels were lower than 100 pg/ml (Fig. 2). Accordingly, TNF production by strains of *M. ulcerans* of intermediate cytotoxicity/virulence was intermediate (Fig. 2). To investigate if the reduced production of TNF by macrophages infected with mycolactone A/B-producing *M. ulcerans* strains was associated with a decreased production of other macrophage-derived proinflammatory cytokines, we evaluated the expression of IL-1 and IL-6. Expression of high amounts of both cytokines occurred in BMDM infected with the mycolactone-negative strain 5114 but not with the mycolactone A/B-producing strain 98-912 (data not shown).

These results show that the production of proinflammatory cytokines such as TNF by BMDM infected with *M. ulcerans* is dependent on the strain's mycolactone-associated toxicity.

The reduced production of TNF by BMDM infected with mycolactone A/B-producing strains is not related to an early cytotoxic effect or to cell death. It has been previously shown that at high MOI, mycolactone-producing *M. ulcerans* strains exhibit an early cytotoxic effect on cultured cells (13, 84). However, the damage to monolayers was found to be prevented when the experimental infection was performed at a low MOI (84).

To evaluate if the inhibition of TNF production by the mycolactone A/B-producing strains was due to an early cytotoxic effect on BMDM, monolayers were infected with different MOIs of *M. ulcerans* 98-912 or *M. ulcerans* 5114, as a control. As shown in Fig. 3A, production of high amounts of TNF was found at high MOIs in macrophages infected with *M. ulcerans*

5114, which diminished as the mycobacterium/macrophage ratio decreased. In contrast, infection with strain 98-912 was not associated with high amounts of TNF production by macrophages, even at a very low MOI (Fig. 3B), for which cytotoxic activity was not observed (data not shown). Additionally, we did not find significant levels of apoptosis in infected macrophages 24 h after infection and macrophages were metabolically active, since they could phagocytose latex beads (data not shown). These results suggest that the very low production of TNF by BMDM infected with the highly virulent strain *M. ulcerans* 98-912 is not associated with an early death of macrophages but possibly to an inhibitory effect of mycolactone.

Addition of mycolactone A/B to BMDM infected with a mycolactone-defective strain inhibits or completely suppresses the production of TNF in a dose-dependent manner. To address the contribution of mycolactone to the inhibition of TNF production by *M. ulcerans*-infected BMDM, we performed experiments using isogenic strains: the WT, mycolactone A/B-producing strain *M. ulcerans* 1615, which is virulent for mice (13), and the isogenic mycolactone-deficient, nonvirulent strain, *M. ulcerans* 1615A (32).

In Fig. 4A, we show that, as previously found for the mycolactone-negative strain 5114 (Fig. 2 and 3), high levels of TNF were produced by macrophages infected with the mycolactone-defective strain 1615A compared to WT, mycolactone-positive *M. ulcerans*. The high levels of TNF produced by macrophages infected with the mutant strain were particularly significant for MOIs of 1:1 or higher (Fig. 4B). On the contrary, much lower amounts of TNF were produced by macrophages infected with WT *M. ulcerans* 1615 at high MOI (Fig. 4C). In fact, for a 1:1 MOI, the reduction of the amount of TNF produced was 4.5-fold, but at an MOI of 5:1 the reduction was 33-fold, comparing the WT with the mutant strain. These results strongly suggest that mycolactone plays a major role in the inhibition of TNF production by *M. ulcerans*-infected BMDM in a dose-dependent manner. To confirm if this inhibitory effect was in fact mycolactone dependent, we infected BMDM with *M. ulcerans* 1615A in the presence of different amounts of mycolactone A/B. Figure 4D shows that the addition of mycolactone inhibited the TNF production induced by the *M. ulcerans* mutant strain in a dose-dependent manner. The addition of 5 ng of mycolactone, the minimal dose tested, reduced significantly the production of TNF, and the highest dose tested (50 ng) abrogated the production of this cytokine (Fig. 4D).

Overall, these results demonstrate that mycolactone plays a major role in the inhibition of TNF production by BMDM infected with virulent *M. ulcerans*.

Addition of mycolactone A/B to BMDM infected with a mycolactone-defective strain moderates the production of MIP-2 in a dose-dependent manner but does not abrogate its production. In contrast with previous descriptions, recent results described inflammatory responses with infiltration of neutrophils and mononuclear cells in a high percentage of cases of *M. ulcerans* infection in humans (38, 84). In addition, it was shown in the mouse model that *M. ulcerans* infection induces a persistent inflammatory response, with the recruitment of neutrophils and macrophages to the infection focus (59). We hypothesized that the recruitment of inflammatory cells in response to infections by *M. ulcerans* strains associated with low levels of

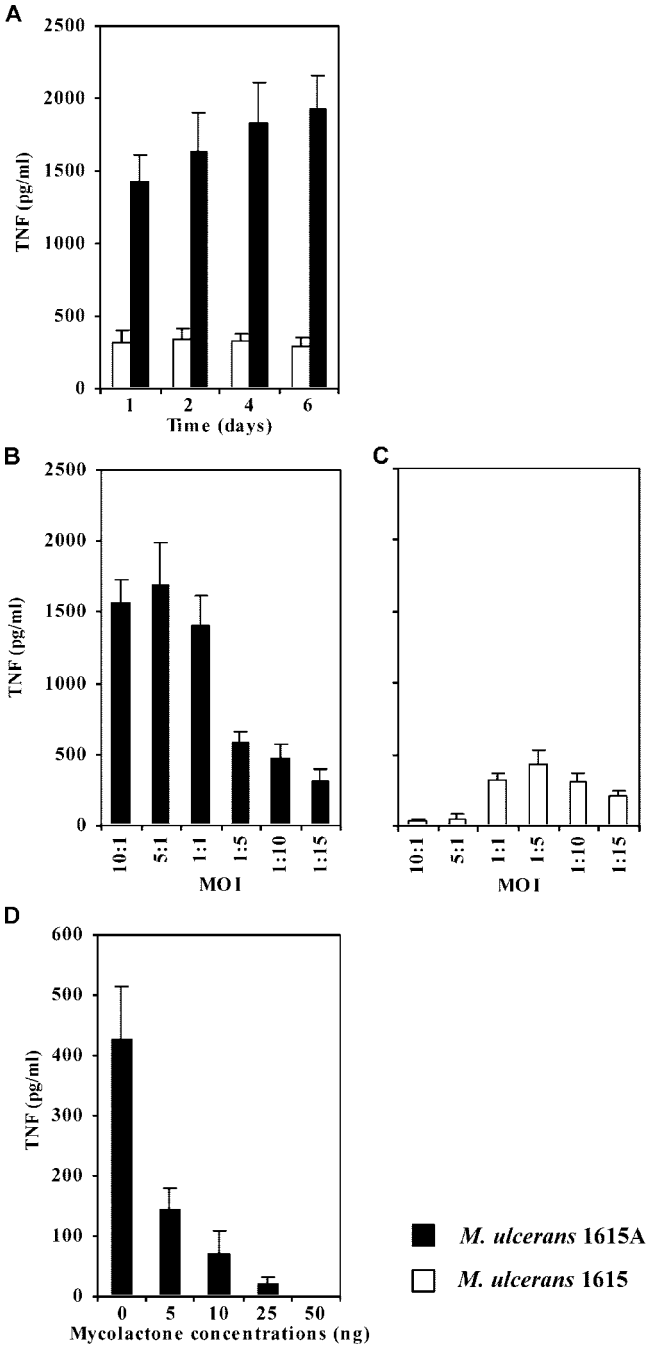

FIG. 4. TNF production by BMDM infected with WT *M. ulcerans* 1615 or with the mycolactone-defective mutant 1615A: kinetics and effects of MOI and supplementation with mycolactone. BMDM were infected with a 1:1 MOI (A and D) or at different MOIs ranging from 10:1 to 1:15 (B and C) with *M. ulcerans* 1615A or 1615 WT, in the presence (D) or absence (A to C) of mycolactone. For cytokine measurement by ELISA, supernatants of three independent wells were collected from each experimental group. (A) Higher amounts of TNF are produced with infection by *M. ulcerans* 1615A in comparison to *M. ulcerans* 1615. (B and C) BMDM infected with *M. ulcerans* 1615A produce large amounts of TNF at high MOIs, and that production declines as the MOI is decreased. *M. ulcerans* 1615 induces the production of smaller amounts of TNF peaking at low MOI. (D) Addition of mycolactone to *M. ulcerans* 1615A-infected macrophages diminishes or abrogates the production of TNF. TNF was not detected in the presence of 50 ng mycolactone. Results are from one representative experiment out of two independent experiments.

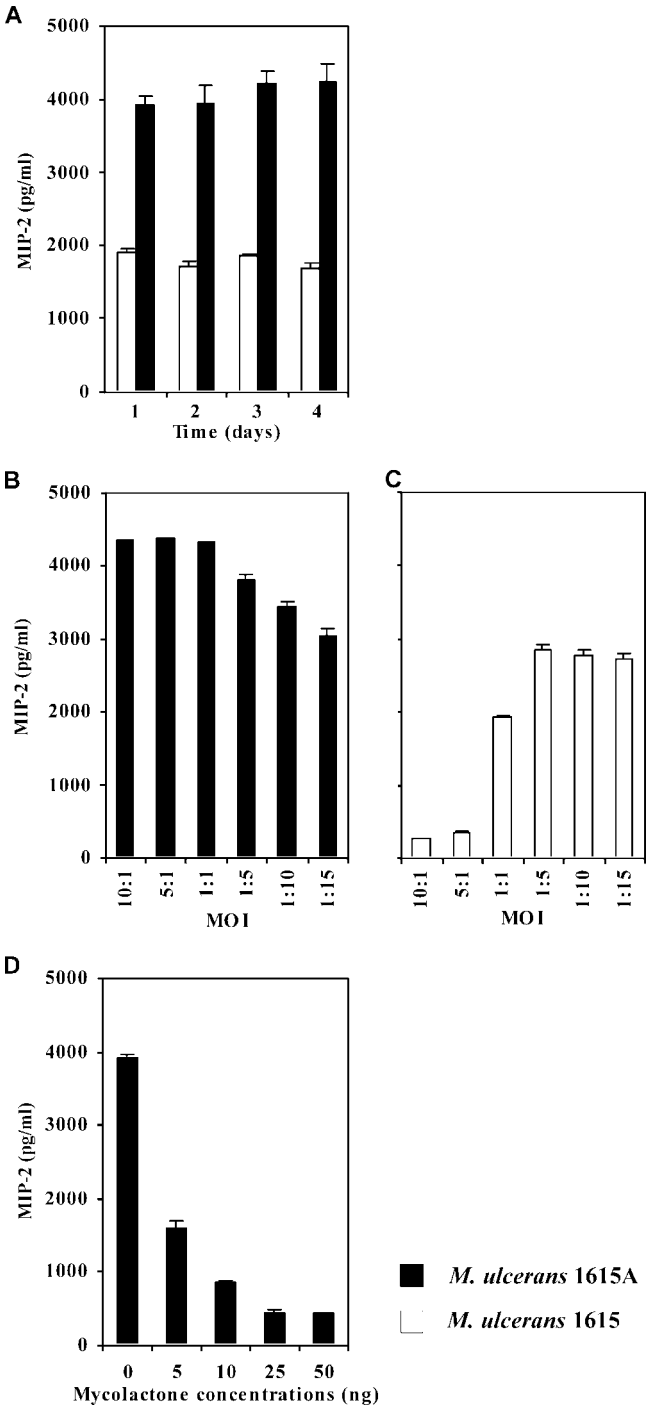

FIG. 5. MIP-2 production by BMDM infected with WT *M. ulcerans* 1615 or with the mycolactone-defective mutant 1615A: kinetics and effects of MOI and supplementation with mycolactone. BMDM were infected with a 1:1 MOI (A and D) or with MOIs ranging from 10:1 to 1:15 (B and C) of *M. ulcerans* strain 1615A or 1615, in the presence (D) or absence (A to C) of mycolactone. For cytokine measurement by ELISA, supernatants of three independent wells were collected from each experimental group. (A) Although *M. ulcerans* 1615A induces larger amounts of MIP-2 in comparison with *M. ulcerans* 1615, significant amounts of the cytokine are produced with the mycolactone-positive strain. (B and C) BMDM infected with *M. ulcerans* 1615A produce large amounts of MIP-2 irrespective of MOI. *M. ulcerans* 1615 induces the production of small but important amounts of MIP-2 at low MOIs. (D) Addition of mycolactone to *M. ulcerans* 1615A-infected macrophages diminishes but does not abrogate the production of MIP-2, even at high concentrations. Results are from one representative experiment out of two independent experiments.

TNF production could be mediated by macrophage-derived chemokines, such as MIP-2. MIP-2 is a chemokine associated with the recruitment of neutrophils and is produced mainly by macrophages (88).

To address this point, we measured the production of MIP-2 following the infection of BMDM with *M. ulcerans* 1615 or 1615A. As shown in Fig. 5A, we found a relevant production of MIP-2 by BMDM infected at a 1:1 MOI, with either strain, although with higher values for the mycolactone-defective strain. For low MOIs, both *M. ulcerans* 1615 as well as the mutant strain defective in mycolactone induced the production of high levels of MIP-2 (Fig. 5B and C). In BMDM infected with the WT strain, low levels of MIP-2 production were only found at high MOIs (Fig. 5C). In fact, at 1:1 and 1:5 MOIs, the reduction of MIP-2 production by BMDM infected with *M. ulcerans* 1615 was 2.2- and 1.3-fold, respectively, compared to the strain defective in mycolactone. Accordingly, the addition of increasing amounts of mycolactone to BMDM cultures only induced a moderate decrease in the production of MIP-2 (Fig. 5D), compared to the effect reported above for TNF production (Fig. 4C). Moreover, this chemokine was still produced in relevant amounts after the addition of the maximal dose of mycolactone tested.

Overall, these results show that the infection of macrophages with a virulent strain of *M. ulcerans* induces the production of important amounts of MIP-2, even at a high MOI, which is in accordance with the occurrence of the inflammatory responses observed in BU patients and experimentally infected mice.

TNF plays a role in the control of infections by *M. ulcerans*. Studies in the mouse model have shown that TNF is a critical cytokine for protective immune responses against *M. tuberculosis* (25). This cytokine has been reported to be produced in BU lesions (64, 66); however, its biological relevance following *M. ulcerans* infection is not known.

Following the characterization of TNF production by macrophages infected with *M. ulcerans*, we tested the biological role of this cytokine in an experimental model of BU. WT or TNF-P55 KO mice were infected with different strains of *M. ulcerans*. The proliferation of mycobacteria in the infected footpads was monitored for a period of 3 months. The growth of strain 5114, which was found to be a strong inducer of TNF, was hindered in vivo by WT mice but not by TNF-P55 KO mice (Fig. 6). For this *M. ulcerans* strain, the increase in the bacterial proliferation between TNF-P55 KO and WT mice was of 0.8 $\log_{10}$ over an 85-day period. In contrast, *M. ulcerans* 98-912, which is not associated with the production of relevant amounts of TNF in vitro, showed similar patterns of mycobacterial proliferation in both mouse strains, before the emergence of ulceration (Fig. 6). Interestingly, we found an intermediate increase of 0.5 $\log_{10}$ in bacterial counts, at day 40 postinfection, between TNF-P55 KO and WT mice infected with strain 94-1327 (Fig. 6), for which an intermediate production of TNF was seen in vitro, as shown above (Fig. 2).

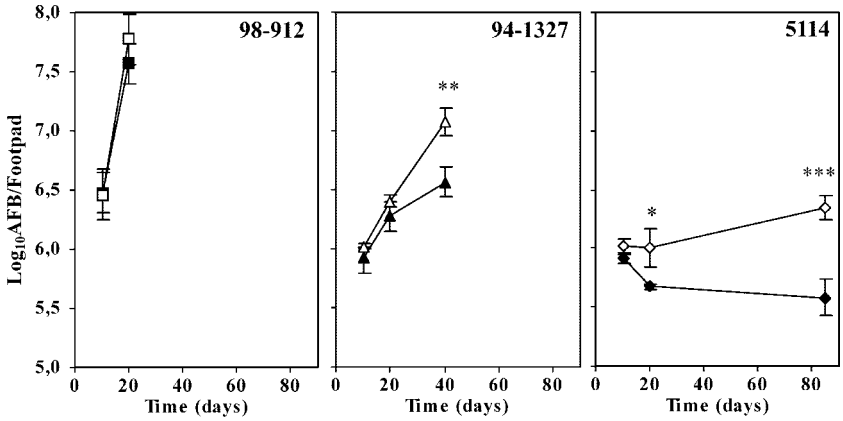

FIG. 6. Proliferation of the mycolactone A/B-producing strain 98-912, mycolactone C-producing strain 94-1327, or mycolactone-negative strain 5114 in wild-type and TNF receptor-deficient mice. Mice (WT [closed symbols] or TNF-P55 KO [open symbols]) were infected subcutaneously in the left hind footpad with 5.3 $\log_{10}$ AFB of *M. ulcerans* 5114 (diamonds), 94-1327 (triangles), or 98-912 (squares). The number of AFB in homogenates from four footpads in each infected group was counted at the indicated time points. WT mice control the proliferation of *M. ulcerans* 5114 but do not eliminate the infection, whereas TNF-P55 KO mice are more susceptible to this strain. An increase in bacterial counts was also found in the footpads of TNF-P55 KO mice infected with strain 94-1327 compared with the WT mice. No significant differences were found for mice infected with *M. ulcerans* 98-912. For ethical reasons, mice were sacrificed after the emergence of ulceration. Statistical differences were determined by comparing TNF-P55 KO mice with WT mice. Calculations were performed using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Results are from one representative experiment out of two independent experiments.

Overall, these results show that TNF plays a protective role in *M. ulcerans* infection.

DISCUSSION

The histopathology of BU lesions in patients infected with *M. ulcerans* has been characterized by a description of minimal or absent cellular inflammation (1, 9, 11, 12, 22, 31–33, 35–37, 40, 41, 56, 65, 75), in contrast with what is known to occur in response to infections by other pathogenic mycobacteria (6, 10, 14, 19, 47, 55, 62, 70, 71, 74, 85). Such a histopathological hallmark has been associated with the *M. ulcerans* toxin mycolactone that, among other effects, inhibits in vitro the production by activated macrophages of the proinflammatory cytokine TNF (13, 60). However, recent publications have demonstrated that *M. ulcerans* induces cellular inflammatory responses in specific areas of infection foci, both in BU patients (38) and in experimentally infected mice (59), with persistence of recruited phagocytes at the periphery of mycolactone-induced necrotic acellular areas (59, 84). These data suggest that infection with viable *M. ulcerans* organisms induces the production of cytokines and/or chemokines responsible for the recruitment and, possibly, activation of inflammatory cells.

In the present work we show that the levels of TNF production by BMDM infected with different strains of *M. ulcerans* are dependent on the cytotoxicity/virulence of the strain. Production of TNF by macrophages was low, intermediate, or high when they were infected with high, intermediate, or noncytotoxic/nonvirulent strains, respectively. The decreased production of TNF by BMDM infected with highly cytotoxic/virulent strains of *M. ulcerans* is not associated with a premature death of macrophages in culture. Indeed, that production was quite scant from the early time points of infection with these strains, as well as in macrophage cultures infected with low MOIs that did not induce cytotoxicity. Macrophages infected with the highly virulent strains can, however, produce relevant amounts of MIP-2, and the addition of mycolactone to cell cultures infected with a mycolactone-defective strain does not abrogate MIP-2 production. These results indicate that the decreased production of TNF during intracellular infection with *M. ulcerans* is not due to a general toxic effect of mycolactone.

MIP-2 is the murine homologue of human IL-8 and plays a central role in the recruitment of neutrophils in mice (27, 88). This chemokine was previously shown to be induced in mice infected with *M. tuberculosis* (68). Recently, the expression of IL-8 was also reported in the lesions of patients infected with *M. ulcerans* (64). The expression of this chemokine in BU lesions is in accordance with the results from Guarner et al. (38) and our own results (84) describing inflammatory infiltrates containing neutrophils in the majority of the BU specimens studied. Coutanceau and coworkers recently showed that a mycolactone-producing strain of *M. ulcerans* induces expression of MIP-2 in infected macrophages (13). These and our present results explain the occurrence of persistent inflammatory infiltrates containing neutrophils in mice experimentally infected with virulent strains of *M. ulcerans* (13, 59). In addition, it cannot be excluded that dead *M. ulcerans*, or bacilli not producing mycolactone, may stimulate MIP-2 production and contribute to the inflammatory response.

Our findings on the levels of virulence for mice, as well as on the cytotoxic activity for infected macrophages, among *M. ulcerans* organisms that are mycolactone A/B or mycolactone C producers are in accordance with previous reports showing a higher cytotoxic effect of purified mycolactone A/B compared to mycolactone C (56). It is clear from our data that the outcome of infection by a particular *M. ulcerans* strain depends on the type of mycolactone secreted by the pathogen. However, other factors contributing to the mycolactone-dependent virulence of *M. ulcerans* cannot be discarded, namely, differences in the amount of toxin produced by each strain and even

the possible regulation of toxin production that could be switched on/off during defined periods of the pathogen's life cycle in the host, as previously discussed (84). In addition, the possible existence of other *M. ulcerans* virulence factors cannot be discarded. Further work is therefore necessary to elucidate the causes for the variability in virulence found among *M. ulcerans* strains.

As discussed above, the association between the mycolactone profile and virulence in mice correlates inversely with the capacity of *M. ulcerans*-infected BMDM to produce TNF. Decreased production of TNF in response to virulent mycobacteria has been previously reported (28, 69). It is known, for instance, that virulent *M. tuberculosis* H37Rv inhibits the production of TNF by infected macrophages to a higher extent than the nonvirulent strain H37Ra (23). However, the mechanisms for the decreased TNF production by these mycobacterial species are still unknown.

M. ulcerans is the only *Mycobacterium* known to produce a cytotoxic exotoxin capable of inhibiting IL-2 and TNF production from phorbol myristate acetate-ionomycin-activated T-cell lines and lipopolysaccharide-activated human monocytes, respectively (60). In the present work, we expand these observations by using a model of infection of primary macrophages with live bacteria from a panel of *M. ulcerans* strains that produce different amounts and types of mycolactone and that exhibit diverse degrees of virulence for mice. The capacity of virulent, mycolactone-producing *M. ulcerans* strains to downregulate the production of TNF might have consequences for the outcome of infection, given that it has recently been showed that *M. ulcerans* has a phase of intramacrophage residence and proliferation (84). In fact, mice deficient in TNF or in its P55 receptor are very susceptible to *M. tuberculosis* infections (4, 26). From studies in the mouse model, it is known that TNF is required to upregulate the macrophage bactericidal mechanisms, particularly in concert with IFN-γ (7, 24, 52). Our present data demonstrate that in experimental *M. ulcerans* infection, the production of TNF also plays a protective role. By inhibiting the production of the macrophage-activating cytokine TNF, virulent *M. ulcerans* strains would be protected from the microbicidal mechanisms of macrophages, allowing an extended time of intracellular proliferation until a significant bacterial load is achieved.

The present and previous results (13, 60) show that mycolactone has an inhibitory activity on cytokine production in vitro. It is still not clear whether this is an early manifestation of impending cell death or a specific suppression of cytokine production. Adusumilli and coworkers recently showed that very low concentrations of mycolactone induce apoptosis in a culture cell line (2). However, more studies are required to address this question in vivo. The presence of TNF in the lesions of BU patients has been inferred by the detection of mRNA for this cytokine in samples of nonulcerative and ulcerative lesions (63, 64, 66), although a high variability in TNF message has been detected in different patients (64, 66) as well as in different areas within the same lesion (63).

These results show that production of TNF in BU lesions can occur despite the presence of mycolactone, which may be associated with bacilli or free in specific areas of the lesions. In this context it is relevant to take into consideration that the spatial relationship of TNF with the cytological pattern in specific areas of a BU lesion, namely, the presence of macrophages and *M. ulcerans* bacilli, is still not clear (63). We can hypothesize that TNF would be produced in BU lesions by (i) macrophages infected with *M. ulcerans* bacilli in which mycolactone production would be temporarily switched off, (ii) macrophages or other cell types distant from mycolactone-producing bacilli in areas not reached by the toxin but in contact with mycobacterial molecules, and (iii) macrophages or other cell types activated by proinflammatory molecules derived from the process of tissue necrosis, regardless of the contact with *M. ulcerans* or *M. ulcerans*-derived molecules.

It has been reported that the process of healing of necrotic ulcers is associated with a granulomatous histopathology (1, 40, 41, 78, 86) and, in contrast, that disseminated disease and osteomyelitis are associated with defects in granuloma formation (42, 78). It is well known that TNF has an important role in the development and maintenance of the granuloma, a key structure that prevents mycobacterial dissemination (4, 21, 26, 30, 48). The recently reported positive correlation between the expression of proinflammatory cytokines, including TNF, and the formation of granulomas in BU lesions (64) supports our interpretation that TNF plays a protective role in *M. ulcerans* infections and that a mycolactone-associated decreased production of that cytokine would occur in the active phase of the infection.

In conclusion, the data presented here show that macrophages infected with virulent, mycolactone-positive strains of *M. ulcerans* produce amounts of the macrophage-activating cytokine TNF that depend on the strain's toxigenesis and that this cytokine contributes to the protection of the infected host against experimental *M. ulcerans* infection.

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CHAPTER 4

Interferon-gamma plays a protective role in *Mycobacterium ulcerans* infection through the activation of nitric oxide production and *LRG-47* expression.

Egídio Torrado, Alexandra G. Fraga, Jenny Carmona, António G. Castro, and Jorge Pedrosa

Original Manuscript

Interferon-gamma plays a protective role in *Mycobacterium ulcerans* infection through the activation of nitric oxide production and *LRG-47* expression.

Running Title: IFN-γ in experimental *M. ulcerans* infection

EGÍDIO TORRADO, ALEXANDRA G. FRAGA, JENNY CARMONA, ANTÓNIO G. CASTRO, AND JORGE PEDROSA*

Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal, [1]

* Corresponding author. Mailing address: Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Portugal. Phone: +351-253-604813. Fax: +351-253-604809, E-mail: jpedrosa@ecsaude.uminho.pt

ABSTRACT

Not much is known about the immune response against *Mycobacterium ulcerans,* the etiological agent of Buruli ulcer (BU). The unique pathology caused by infections with this pathogen is closely associated with the production of the lipidic exotoxin mycolactone. Following the demonstration of an intramacrophage growth phase of *M. ulcerans* in the host, we investigated the biological relevance of interferon-gamma (IFN-γ) in vivo during an experimental *M. ulcerans* infection, as well as the protective mechanisms activated by this cytokine to control the intramacrophage proliferation of several *M. ulcerans* strains with different cytotoxicity/virulence for mice.

We herein show that IFN-γ-deficient mice are more susceptible than wild-type mice to infection by nonvirulent and intermediate virulent strains of *M. ulcerans* but not to infection by a highly virulent strain. In accordance with the in vivo data, IFN-γ-activated cultured macrophages control the proliferation of the nonvirulent and intermediate virulent strains at a low multiplicity of infection. For the nonvirulent strain, the protective mechanism activated in macrophages was nitric oxide (NO)-dependent, since competitive-inhibition of the Nitric Oxide Synthase renders macrophages unable to control the proliferation of that strain. In contrast, NO production was not found in macrophages infected with the intermediate or highly virulent strains of *M. ulcerans*. However, macrophages infected with nonvirulent or intermediate virulent strains expressed high levels of *LRG-47*, a gene associated with phagosome maturation. These results suggest that intracellular *M. ulcerans* producing mycolactone interfer with the IFN-γ-dependent activation of macrophages that is responsible for the initiation of NO production and phagosome maturation required to control *M. ulcerans* proliferation.

INTRODUCTION

Previous work from our laboratory has shown that, like other pathogenic mycobacteria, *Mycobacterium ulcerans* has a phase of intramacrophage proliferation (28). After the initial phase of intracellular residence and proliferation, lasting for a period of time that depends on the cytotoxicity of the strain, *M. ulcerans* kills the host cell becoming extracellular (28). Lysis of host cells, as well as destruction of tissues in infection foci is closely associated with the production of the *M. ulcerans* exotoxin mycolactone (10). Mycolactone was shown to passively diffuse through the plasmatic membrane of cells (24) inducing apoptosis and necrosis (1), having therefore an important impact on disease progression. Mycolactone is indeed a key pathogenic factor in Buruli ulcer (BU), namely by inducing death of inflammatory cells (28) recruited during *M. ulcerans* infection (13, 18) and by inhibiting tumor necrosis factor (TNF) production (19, 27). Therefore, the host immune response to *M. ulcerans* is determined by the mycobacterial nature of the pathogen compounded by the secretion of mycolactone.

It is known that a protective immune response is mounted in individuals resistant to *M. ulcerans* that ultimately leads to the control of *M. ulcerans* without clinical manifestations (5). In addition, in a study of clofazamine treatment in patients with nodular lesions, the authors reported that 30% of nodules in the placebo group healed spontaneously (22). However, not much is known about the nature of the protective immune response against *M. ulcerans* infections.

Different authors have reported the expression of several cytokines in BU lesions, including interferon-gamma (IFN-γ) (11, 12, 20, 21). This cytokine was firstly suggested to play a role in the control of *M. ulcerans* infections by Gooding and colleagues (11, 12). These authors found that peripheral blood mononuclear cells (PBMC) 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* BCG bacilli. In a posterior study, Westenbrink and colleagues found that, compared to matched community controls, PBMC from BU patients produce significantly higher levels of IFN-γ in late stages of the disease but not in early stages, when PBMC were stimulated with purified protein derivative (PPD) (30). When PCR analyses was used to quantify the expression of this cytokine in nodular or ulcerative BU lesions, Prevot and colleagues reported a higher expression of IFN-γ in the nodular lesions while a lower expression was detected in the more severe ulcerative lesions (21). On the other hand, in a more recent study, Phillips and colleagues reported a strong expression of IFN-γ in both nodular and

ulcerative lesions (20). Although with discrepant results, the reports discussed above suggest a protective role for IFN-γ in BU.

IFN-γ is mainly produced by both $CD4^+$ and $CD8^+$ T cells and natural killer cells (8, 15). Being a powerful activator of the macrophage microbicidal mechanisms, IFN-γ is a key cytokine in the control of intracellular mycobacterial infections (8, 15). IFN-γ and TNF-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 *M. tuberculosis* growth arrest (8). In addition, IFN-γ and TNF activate nitric oxide synthase 2 (NOS2) to produce nitric oxide (NO), a powerful microbicidal molecule (8). In IFN-γ deficient mice, *Mycobacterium tuberculosis* grows unchecked, being this host extremely susceptible to this pathogen (4, 9). Lack of macrophage activation and NOS2 expression are the likely factors that contribute to the extreme susceptibility of these IFN-γ deficient mice (9).

As discussed above, and although IFN-γ has been suggested to be associated with resistance to *M. ulcerans* infections (11, 12, 20), there is no direct evidence for a protective role of this cytokine in BU. The recent demonstration of an intramacrophage growth phase of *M. ulcerans* (28), directed us to study the role of IFN-γ in experimental *M. ulcerans* infections. Therefore, the objective of the present work was to investigate the biological relevance of IFN-γ in *M. ulcerans* infections and to dissect the anti-microbial mechanisms activated by this cytokine in macrophages infected with *M. ulcerans*.

Our results show that IFN-γ is expressed in mouse *M. ulcerans*-infected footpads. In addition, the increased proliferation in IFN-γ-deficient mice of the mycolactone C-producing, intermediate virulent strain, *M. ulcerans* 94-1327 shows that IFN-γ induced during *M. ulcerans* infection is protective for the host. In vitro, IFN-γ-activated macrophages control the proliferation of the mycolactone-negative mutant strain 5114 as well as the virulent strain 94-1327. For the nonvirulent *M. ulcerans* 5114 strain, the bactericidal activity of activated macrophages is NO-dependent. On the other hand, no increased NO production was detected in IFN-γ-activated macrophages infected with the mycolactone-producing strains *M. ulcerans* 94-1327 or 98-912. However, *LRG-47* expression was detected in IFN-γ-activated macrophages infected with *M. ulcerans* 5114 and 94-1327, indicating enhanced phagosome maturation. These results are in accordance with previous data suggesting the involvement of cell-mediated immunity and delayed-type hypersensitivity in human and experimental BU, and provide a mechanism by which *M. ulcerans* proliferation is controlled in the host.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *M. ulcerans* strains used in this study were selected based on their geographical origin and on the type of mycolactone produced. Strain 98-912, a highly virulent strain (18, 27), was isolated from a Chinese patient and produces a mycolactone A/B slightly different from those in African strains (14). Strain 94-1327, an intermediate virulent strain (27), is an isolate from Australia and produces the characteristic mycolactone C (17) since 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 (25). Strain 5114, a nonvirulent strain (18, 27), is an isolate from Mexico and does not produce mycolactone (17) due to the loss of key genes involved in the synthesis of this macrolide (25). All strains used in this work are from the collection of the Institute of Tropical Medicine (ITM), Antwerp, Belgium.

The isolates were grown on solid 7H9 medium at 32°C for approximately 1 month, recovered, diluted in PBS to a final concentration of 1 mg/ml and vortexed using 2-mm glass beads. The number of acid-fast bacilli (AFB) in the inocula was determined according to the method described by Shepard and McRae (23). The final suspensions revealed more than 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 (Barcelona, Spain) and were housed in specific pathogen-free conditions with food and water *ad libitum*.

Footpad model of 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. The right hind footpad was used as a control. At different time points after infection, *M. ulcerans* growth in footpad tissues of infected mice was evaluated as previously described (18). Briefly, tissue specimens were carefully minced on a petri dish. The tissues were resuspended in 2 ml PBS containing 0.04% Tween 80 (Sigma) and vortexed vigorously with 2-mm glass beads to obtain homogenized suspensions. At different times postinfection, AFB counts were performed on samples from five footpads in each group by using the method of Shepard and McRae (23).

Culture of murine bone marrow-derived macrophages (BMDM). 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 cold Hanks' balanced salt

solution (HBSS) (Gibco, Paisley, United Kingdom). The resulting cell suspension was centrifuged at 500 x *g* and resuspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Gibco), 10 mM glutamine (Gibco), 10% heat-inactivated fetal bovine serum (Sigma), and 10% L929 cell conditioned medium (complete DMEM [cDMEM]). To remove fibroblasts or differentiated macrophages, cells were cultured for a period of 4 hours on cell culture dishes (Nunc, Naperville, IL) with cDMEM. Nonadherent cells were collected with warm HBSS, centrifuged at 500 x *g*, distributed in 24-well plates at a density of 5 x 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 days 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 (18).

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 in order to obtain a multiplicity of infection (MOI) indicated for each experiment (bacteria/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, in cDMEM with 100 Uml^{-1} of recombinant IFN-γ or in cDMEM with 100 Uml^{-1} of recombinant IFN-γ and NG-monomethyl-L-arginine (L-NmmA), an inhibitor of nitric oxide synthases (NOS) or NG-monomethyl-D-arginine (D-NmmA), 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 (23).

At different time points after infection, bacterial growth was assessed by counting colony forming units (CFU) from macrophage monolayers. Briefly, at the selected time point, macrophage monolayers were lysed with saponin (0.1% final concentration), and serial dilutions were seeded in 7H9 agar medium with OADC supplement. Plates were then incubated at 32°C for 6 to 8 weeks.

Nitrite quantification. Nitrite production by the macrophage monolayers was determined by the Griess assay as described elsewhere (26). Briefly, macrophage supernatants were thawed and diluted in a 96-well enzyme-linked immunosorbent assay plate (Nunc), and an equal

volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine, 2.5% H_3PO_4) was added. The absorbance at 550 nm was measured on an enzyme-linked immunosorbent assay reader, and the concentration of nitrite was calculated by comparing optical density values to a standard curve of $NaNO_2$ in cDMEM.

Quantitative RT-PCR analysis. Total RNA from 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 (*HPRT*) and *LRG-47*. The LightCycler-FastStart DNA Master Hybridization Probes mixture was used according to the manufacturer's instructions. The HPRT reaction mixture included 5 mM Mg^{2+}, sense primer (5'-GCTGGTGAAAAGGACCTCT-3'), anti-sense primer (5'-CACAGGACTAGAACACCT GC-3') and the gene specific probes 5'-LC red640-TCTGCAAATACGAGGAGTCCTGTTG$_P$-3' and 5'-AAAGCCTAAGATGAG CGCAAGTTGA$_F$-3'. The cycling parameters were 1 cycle of 95°C for 10 min, followed by 45 cycles of 94°C for 10 sec, 60°C for 10 sec and 72 °C for 11 sec. The temperature transition rate was 20°C. The *LRG-47* reaction mixture included 5 mM Mg^{2+}, sense primer (5'-CTCTGGATCAGGGTTTGAGGAGTA-3'), anti-sense primer (5'-GGAACTGTGATGGTTTCATGATA-3') and the gene specific probes 5'-LC red640- AGGTCCACAGACAGCGTCACTCGG$_P$-3' and 5'-AACCAGAGAGCCTCACCAGGGAGCTGA$_F$-3'. The cycling parameters were 1 cycle of 95°C for 10 min, followed by 45 cycles of 94°C for 10 sec, 58°C for 10 sec and 72 °C for 20 sec.

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

IFN-γ is induced in murine footpads infected with either a mycolactone-negative mutant or mycolactone-producing strains of *Mycobacterium ulcerans*. Interferon-gamma (IFN-γ) production by the host is a hallmark of protection in infections by intracellular parasites, including *Mycobacterium avium* (2, 7) and *Mycobacterium tuberculosis* (4, 9). The role of IFN-γ in *M. ulcerans* infection was not experimentally addressed so far, even though some authors have suggested that resistance to *M. ulcerans* infection in humans is associated with IFN-γ production (11, 12, 20).

To study the kinetics of IFN-γ expression following infection by *M. ulcerans*, mice were infected in the left hind footpad with different strains of *M. ulcerans*, which do not produce mycolactone or produce different types of mycolactone, showing different degrees of virulence for mice (18, 27). As represented in FIG. 1, all *M. ulcerans* strains tested induced the in vivo expression of IFN-γ following infection by the subcutaneous (s.c.) route. Nonetheless, the mycolactone-deficient *M. ulcerans* 5114 induced a higher expression of this cytokine, with a peak of expression at day 20 after infection (FIG. 1). The highly virulent, mycolactone-producing *M. ulcerans* 98-912 induced a low but sustained level of expression of IFN-γ with similar kinetics as compared with *M. ulcerans* 5114.

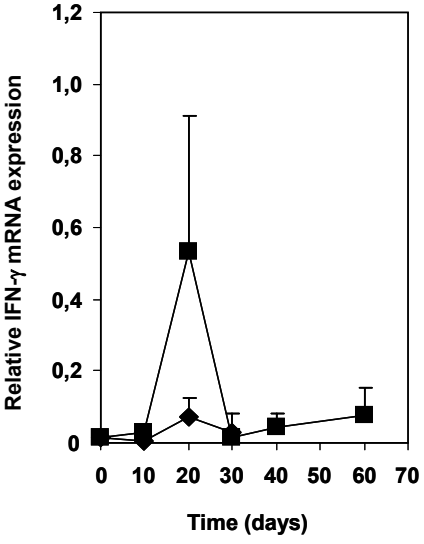

FIG. 1 – Infection with *M. ulcerans* 5114 or 98-912 induces the expression of IFN-γ in infected footpads. Mice were infected subcutaneously with 5.3 $\log_{10}$ of *M. ulcerans* 5114 (squares) or 98-912 (diamonds) in the left hind footpad. Noninfected mice were used as controls. At different times postinfection, footpad cells were harvested, mRNA extracted and IFN-γ expression was evaluated by Real Time-PCR. Both *M. ulcerans* strains induce IFN-γ in the infected footpad; however, the nonvirulent *M. ulcerans* strain 5114 induces higher levels of IFN-γ expression when compared with *M. ulcerans* 98-912.

These results show that, like in human BU lesions (11, 12, 20), IFN-γ message is detected in the infectious focus of experimental infections with mycolactone-deficient or mycolactone producing strains of *M. ulcerans*. Additionally, the levels of IFN-γ message were

found to be different following infection with the different *M. ulcerans* strains. It is possible that the induction of IFN-γ is related with the virulence of the infecting strain, being low virulent strains high inducers of IFN-γ and highly virulent strains low inducers of this cytokine. These results could explain the incongruent results reported in the literature, regarding the expression of IFN-γ in Buruli ulcer (BU) lesions (20, 21). Indeed, among the different studies carried out in BU samples to elucidate the role of different cytokines in BU, there is no information on the cytotoxicity/virulence of the infecting strains. It is therefore possible that the variation in IFN-γ expression found in different studies (30) is associated with different degrees of virulence of the infecting strains.

IFN-γ plays a role controlling *M. ulcerans* proliferation both in vivo and in vitro. Taking into consideration the fact that both the mycolactone-negative mutant and the mycolactone-producing strains of *M. ulcerans* induced IFN-γ expression after infection by the s.c. route, we evaluated the role of this cytokine in experimental infections by *M. ulcerans*, in vivo and in vitro.

To address the biological relevance of IFN-γ in an experimental model of BU, we compared the proliferation of the three *M. ulcerans* strains in the footpad of wild-type (WT) or IFN-γ-deficient mice. The proliferation of mycobacteria in infection foci was monitored by counting acid-fast bacilli (AFB) in homogenates of the infected footpads at selected time points. As represented in FIG. 2A, the proliferation of *M. ulcerans* 5114 was controlled by WT mice but not by IFN-γ-deficient counterparts, with a difference of 1.47 $\log_{10}$ AFB at day 245 of experimental infection. In contrast, no differences were found in the proliferation of the highly virulent strain *M. ulcerans* 98-912 in WT or IFN-γ-deficient mice even when low infectious doses were used (FIG. 2C). Interestingly, IFN-γ-deficient mice, were slightly more susceptible to the infection with the intermediate virulent *M. ulcerans* strain 94-1327 when compared with WT mice, with an increased proliferation of 0.4 $\log_{10}$ AFB over the first 35 days of infection (FIG. 2B). After this time point, this strain was found not to be controlled by either WT or IFN-γ-deficient mice.

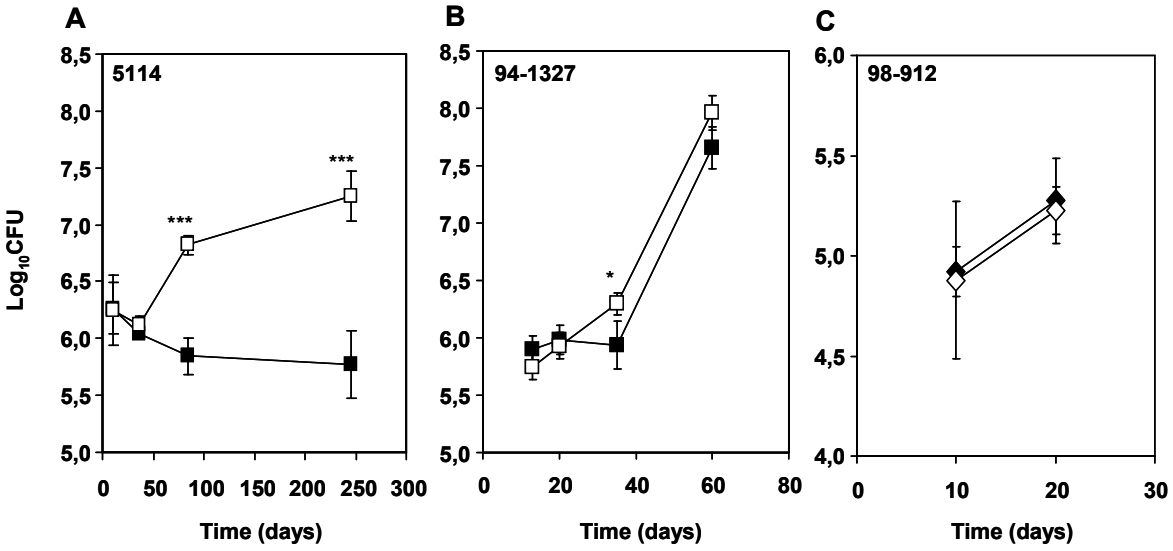

FIG. 2 – IFN-γ-deficient mice have an increased susceptibility to *M. ulcerans*. Mice (WT [closed symbols] and IFN-γ-deficient [opened symbols]) were infected subcutaneously in the left hind footpad with 5 $\log_{10}$ AFB of *M. ulcerans* 5114 or *M. ulcerans* 94-1327 or 4 $\log_{10}$ AFB of *M. ulcerans* 98-912. The number of AFB in homogenates from four footpads in each infected group was counted at the indicated time points. Statistical differences were determined by comparing IFN-γ-deficient mice with WT mice. Calculations were performed using Student's *t* test. *, $p<0.05$; ***, $p< 0.001$.

WT mice control the proliferation of *M. ulcerans* 5114 but do not eliminate the infection, whereas IFN-γ-deficient mice are susceptible to the infection with this strain, with a difference of 1.47 log10 AFB at day 245. An increased bacterial burden was also found in the footpads of IFN-γ-deficient mice infected with strain 94-1327, at day 40 after infection, as compared with WT mice. No significant differences were found for mice infected with *M. ulcerans* 98-912.

To further address the role of IFN-γ in BU, and considering our previous demonstration of an intramacrophage growth phase of *M. ulcerans* (28), bone marrow-derived macrophages (BMDM) were infected with the previously described strains of *M. ulcerans* in the presence or absence of IFN-γ. As represented in FIG. 3A, *M. ulcerans* 5114 proliferated inside BMDM over the 8 day-period studied. In accordance with the in vivo results obtained for this strain (FIG. 2A), in the presence of IFN-γ there was a 1.38 $\log_{10}$ reduction of the colony forming units (CFU) counts. On the other hand, no differences were found in the proliferation of the highly virulent strain *M. ulcerans* 98-912 in IFN-γ-activated or nonactivated macrophages (FIG. 3D) regardless of the MOI used to infect the BMDM cultures (data not shown). For BMDM infected with the intermediate virulent strain 94-1327, different results were obtained acording with the tested multiplicity of infection (MOI). At an MOI of 1:1, we found that this *M. ulcerans* strain was not controlled either by nonactivated or IFN-γ-activated BMDM (FIG. 3B). However, at the lower MOI

of 1:3, IFN-γ-activated BMDM controlled the proliferation of *M. ulcerans* 94-1327 in the first 4 days of infection, with a 0.2 $\log_{10}$ reduction of the CFU counts (FIG. 3C).

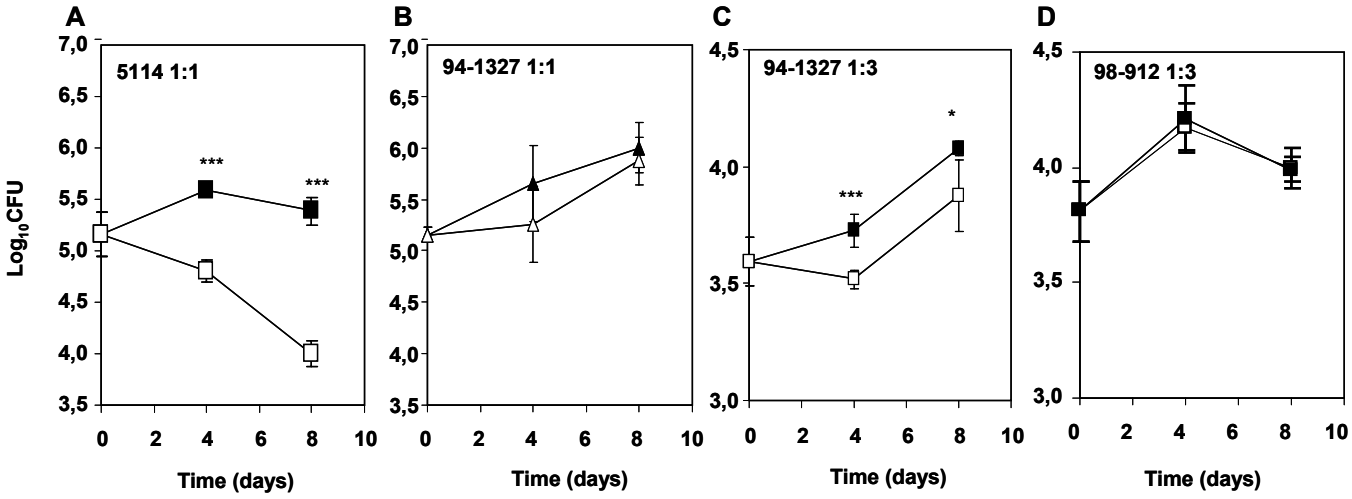

FIG. 3 – IFN-γ-activated macrophages control the proliferation of *M. ulcerans* 5114 and *M. ulcerans* 94-1327 but not *M. ulcerans* 98-912. BMDM infected with *M. ulcerans* 5114 (A) or 94-1327 (B) at a 1:1 MOI or with *M. ulcerans* 94-1327 (C) or 98-912 at a 1:3 MOI (D) were left untreated (closed symbols), or activated with 100 Uml^{-1} of recombinant IFN-γ every other day after infection (opened symbols). At different times postinfection, macrophages were lysed and CFUs were performed. Statistical differences were determined by comparing IFN-γ-activated with nonactivated macrophages. Calculations were performed using Student's *t* test. *, $p<0.05$; ***, $p< 0.001$.
All *M. ulcerans* strains were found to persist in nonactivated macrophages, but IFN-γ-activated macrophages were found to control the proliferation of *M. ulcerans* 5114 as well as *M. ulcerans* 94-1327 at the lower MOI of 1:3. On the other hand, proliferation of *M. ulcerans* 98-912 was similar in both IFN-γ-activated or nonactivated BMDM at the lower MOI of 1:3.

Overall, these results show that IFN-γ plays a protective role in *M. ulcerans* infections, by activating macrophages to control *M. ulcerans* intracellular proliferation.

IFN-γ-activated macrophages infected with *M. ulcerans* 5114 or 94-1327, but not *M. ulcerans* 98-912, show an increased production of NO or *LRG-47* expression. IFN-γ has been shown to increase the bactericidal activity of macrophages by inducing the production of nitric oxide (NO) (6, 8) and phagosome-lysosome fusion (29), as well as by increasing the production of proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) (3). The role of these effector mechanisms in the control of *M. ulcerans* is still not known.

We first evaluated the production of NO, by measuring the accumulation of nitrite in the culture medium, by IFN-γ-activated BMDM following infection by the three different strains of *M. ulcerans*. As represented in FIG. 4, IFN-γ-activated macrophages infected with the mycolactone-negative strain 5114 produced high amounts of nitrite over the 8 days of experimental infection. However, macrophages infected with the mycolactone-producing strains 98-912 or 94-1327 were found not to produce significant amounts of nitrite, even after stimulation with IFN-γ. These results suggest that mycolactone-producing strains of *M. ulcerans* inhibit the production of NO by macrophages activated with IFN-γ.

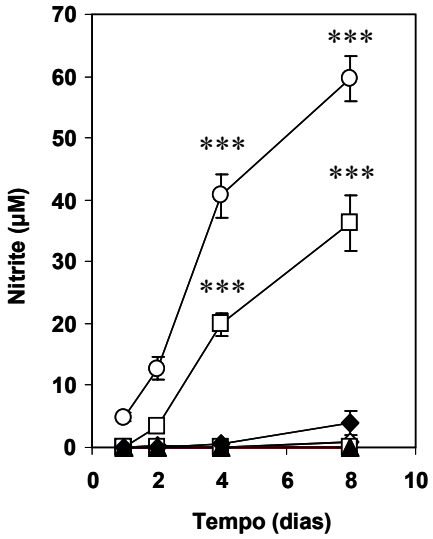

FIG. 4 – IFN-γ-activated macrophages infected with *M. ulcerans* 5114 produce higher amounts of nitrite. BMDM infected with *M. ulcerans* 5114 (squares), 94-1327 (triangles) or 98-912 (diamonds) were left untreated (closed symbols) or activated with 100 Uml^{-1} of recombinant IFN-γ every other day after infection (opened symbols). Noninfected macrophages (opened circles) or macrophages activated with IFN-γ and LPS (closed circles) were used as negative and positive control, respectively. At different times postinfection, levels of nitrite production were assayed in the culture medium by using the Griess method. Statistical differences were determined by comparing IFN-γ-activated with nonactivated macrophages, infected or noninfected. Calculations were performed using Student's *t* test. ***, $p< 0.001$.

IFN-γ-activated BMDM infected with *M. ulcerans* 5114 produce higher amounts of nitrite when compared to the amounts produced by IFN-γ-activated BMDM infected with *M. ulcerans* 94-1327 or 98-912. Noninfected or infected but nonactivated BMDM do not produce nitrite.

The biological relevance of NO in the control of the intramacrophage proliferation of *M. ulcerans* was further evaluated following infection of BMDM with strain 5114 in the presence of the competitive nitric oxide synthase (NOS) inhibitor L-NmmA. The molecule D-NmmA was used as a control. As represented in FIG. 5, the competitive inhibition of NOS reduces the capacity of IFN-γ-activated macrophages to control the infection by this strain of *M. ulcerans*, showing that NO production is essential to control the proliferation of intramacrophage *M. ulcerans* 5114. Interestingly, in the presence of L-NmmA, there is still some control of *M. ulcerans* 5114 proliferation, suggesting that other macrophage microbicidal mechanisms, independent of NO production, are also important to control the intramacrophage proliferation of *M. ulcerans*.

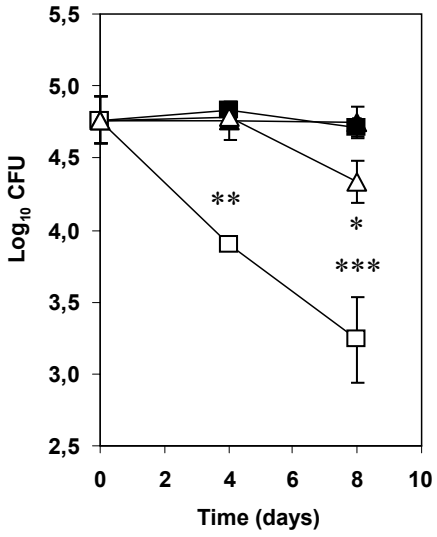

FIG. 5 – Nitric oxide produced by IFN-γ-activated macrophages plays a key role in the control of *M. ulcerans* 5114. BMDM infected with *M. ulcerans* 5114 nonactivated (closed symbols) or activated with 100 Uml^{-1} of recombinant IFN-γ every other day after infection (opened symbols) were treated with L-NmmA (triangles) or, as a control, with D-NmmA (squares). Statistical differences were determined by comparing IFN-γ-activated with nonactivated macrophages for the same condition. Calculations were performed using Student's *t* test.*, $p<0.05$; **, $p<0.01$;***, $p< 0.001$.

BMDM activated with IFN-γ and treated with the NOS inhibitor L-NmmA were unable to control *M. ulcerans* 5114, whereas macrophages treated with D-NmmA killed the bacteria, showing that nitric oxide plays a key role controlling the proliferation of this *M ulcerans* strain.

The phagosome-lysosome fusion is another important mechanism involved in resistance to infection by intracellular parasites (8, 15). *LRG-47* expression was shown to be associated with phagosome maturation (16). Taking this into consideration, we measured the level of expression of *LRG-47* in *M. ulcerans*-infected macrophages. As represented in FIG. 6, IFN-γ-activated BMDM infected with *M. ulcerans* 5114 showed high levels of *LRG-47* expression 12 hours after infection. Interestingly, IFN-γ-activated, *M. ulcerans* 94-1327-infected macrophages showed a decreased expression of *LRG-47* when compared to macrophages activated with IFN-γ alone. On the other hand, there was a complete inhibition of *LRG-47* in activated macrophages infected with *M. ulcerans* 98-912. This set of results suggests that mycolactone-producing *M. ulcerans* strains inhibit the maturation of the phagosome, probably providing these strains a nonacidic environment to survive and proliferate within macrophages. However, this effect seems to be dependent on the type of mycolactone produced by *M. ulcerans*, since the intermediate virulent strain 94-1327, a mycolactone C producer, does not inhibit phagosome maturation at the same extent as the highly virulent *M. ulcerans* 98-912 strain, a mycolactone A/B producer. The higher expression of *LRG-47* by *M. ulcerans* 94-1327-infected macrophages explains the control of this strain by IFN-γ-activated macrophages (FIG 3D). However, this control is not as efficient when compared with *M. ulcerans* 5114, since there is no production of NO by *M. ulcerans* 94-1327 infected, IFN-γ-activated macrophages (FIG. 4).

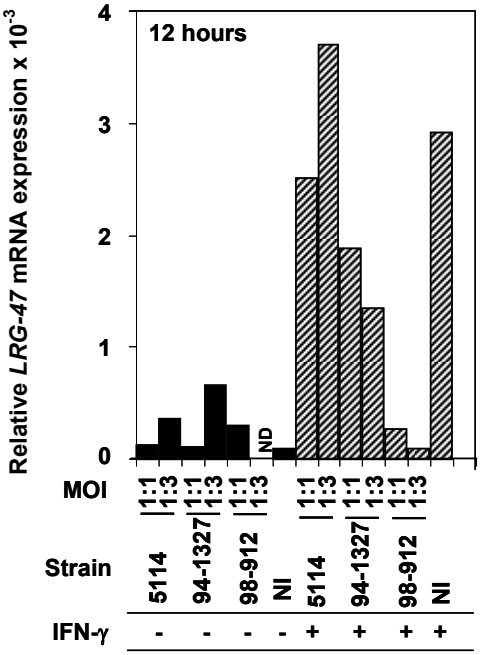

FIG. 6 – Infection of BMDM with *M. ulcerans* 5114 or 94-1327 induces *LRG-47* expression. BMDM infected with *M. ulcerans* 5114, 94-1327 or 98-912 were left untreated (closed bars) or activated with 100 Uml^{-1} of recombinant IFN-γ every other day after infection (dashed bars). Macrophages noninfected, activated or nonactivated (opened bars) with IFN-γ were used as control. At 12 hours after-infection, mRNA was extracted and *LRG-47* expression was evaluated by Real Time-PCR. BMDM infected with *M. ulcerans* 5114, and in a lesser extent, infected with *M. ulcerans* 94-1327 express *LRG-47*, indicative of phagosome maturation. On the other hand, no expression of this gene was detected in BMDM infected with *M. ulcerans* 98-912. ND, not-detected.

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CHAPTER 5

DISCUSSION

5.1. GENERAL DISCUSSION

Buruli ulcer (BU) is a serious skin disease caused by infections with *Mycobacterium ulcerans*. Although this pathogen was described for the first time in 1948 by MacCallum and colleagues (50) in Australia, it has received little attention until the last decade. BU affects inhabitants of different tropical regions, mainly in poor rural areas in Africa where inadequate or no health services are available. All ages and sexes are affected; however most patients are among children younger than 15 years old (5). Since the 1980s, the disease has emerged dramatically in West African countries and, in some communities, has become more prevalent than tuberculosis and leprosy (43).

BU is difficult to treat and can result in serious sequelae, deformities and functional disabilities. Different measures have been suggested to control BU (22). However, associated with this disease, there is a strong social stigma and many communities in Africa see BU as a curse rather than as a disease. For that reason, vaccination to prevent BU would be the most satisfactory strategy to control the disease. Although BCG vaccination to prevent BU was shown to confer some degree of protection, the protection conferred was short lived (76, 84), and thus a specific and more effective vaccine is urgently needed. To develop such a vaccine against BU, it is required to understand the very first stages of the interaction of *M. ulcerans* with the host's immune system, which cannot be studied in the human infection because samples of initial BU lesions are not available.

M. ulcerans is genetically closely related with *Mycobacterium marinum* and *Mycobacterium tuberculosis* (85); however the disease caused by *M. ulcerans* is particular in some key aspects. In fact, unlike other pathogenic mycobacteria, *M. ulcerans* produces a lipidic toxin, mycolactone, that was shown to have a key role in *M. ulcerans* pathogenesis (33). It was shown that injection of purified mycolactone in guinea pig skin induces an ulcer similar to that induced by virulent *M. ulcerans* bacilli (33, 48). Additionally, mycolactone-negative mutant *M. ulcerans* strains were shown to be nonvirulent for mice and guinea pigs (33, 58), supporting the central role played by this toxin in the pathogenesis of BU.

When we started our work on *M. ulcerans* infection, it was not clear whether: (i) *M. ulcerans* was always an extracellular pathogen, as it has been described in the literature, or if there was an intracellular phase in the life cycle of this pathogen; (ii) what cytokines and chemokines were produced upon infection with this pathogen, as well as their relevance to

control *M. ulcerans* proliferation; and (iii) what microbicidal mechanisms were required to prevent the proliferation of this pathogen in the host.

The propose of the present dissertation was to contribute to address these questions, that will be very important to better understand the characteristics of a protective immune response against *M. ulcerans* and, in the future, for the development of a vaccine or better therapeutic strategies.

Intramacrophage growth phase of *M. ulcerans*. The characterization of a bacterial pathogen as an intracellular parasite is based on a set of characteristics: (i) they promote phagocytosis by professional phagocytes, usually macrophages, and express genes that allow them to survive and multiply intracellularly (55, 72), although they usually also have a phase of extracellular multiplication (18, 38); (ii) the pathology caused after infection is associated with their intracellular life style (45, 61); and (iii) the immune response involves cell-mediated immunity (CMI) mechanisms which generally requires cytotoxic T lymphocytes or a T helper (Th)1-type response (45, 61).

We and other groups have shown that *M. ulcerans* is cytotoxic for cultured macrophages (34, 58). Although being intracellular parasites, several mycobacterial species have cytotoxic activity against macrophages or other cell types (49, 69), however, *M. ulcerans* is the most cytotoxic of all known mycobacteria (20). Due to its cytotoxicity, associated with mycolactone production, *M. ulcerans* has been considered unable to grow inside cultured macrophages (3, 70). Moreover, BU lesions are characterized histopathologically by large numbers of extracellular bacilli in necrotic acellular areas (10, 20, 23, 37, 44). These two sets of observations led most investigators to classify *M. ulcerans* as an extracellular pathogen (3, 11, 14, 20, 33, 37, 40, 60).

In what regards the presence of phagocytes in BU infection foci, previous work from our laboratory showed that experimental infections with *M. ulcerans* in the mouse footpad induce a persistent inflammatory response with a profile that is dependent on the cytotoxicity/virulence of the strain (58). The inflammatory infiltrate induced by virulent, mycolactone-producing strains was found at the periphery of the lesion, while large clumps of *M. ulcerans* bacilli were present in a central necrotic acellular area. More importantly, *M. ulcerans* bacilli were consistently found co-localizing with phagocytes in the areas of inflammation (58). Therefore, considering this constant

availability of inflammatory cells to interact with *M. ulcerans*, and that resistance to infections by *M. ulcerans* in humans and mice has been associated with CMI and delayed-type hypersensitivity (DTH) responses (21, 35, 36, 40, 47, 50, 51, 57, 63, 66, 74, 77, 79, 82, 88), led us to consider that the typical histopathology of BU lesions (large numbers of extracellular bacilli in necrotic acellular areas) may not reflect the actual host-parasite interactions at the active foci of infection in patients with BU and that *M. ulcerans*, like other pathogenic mycobacteria, would have an intracellular growth phase in the host. In this context, it is relevant that some early investigators have reported intracellular *M. ulcerans* inside macrophages and neutrophils both in human lesions as well as in experimental models of infection (48, 50, 71).

Therefore, our initial studies were directed toward the evaluation of the interactions between phagocytes and *M. ulcerans* in human infections, interactions that so far have not been analysed in detail.

In our analysis of 19 samples from confirmed BU cases (chapter 2), inflammatory infiltrates were observed in 15, a result that agrees with a recent report describing cellular inflammatory responses in a high percentage of BU cases, although with a minimal extension (39). Eight of the 15 specimens with inflammatory infiltrates and acid-fast bacilli (AFB) revealed intramacrophage *M. ulcerans* in the periphery of the necrotic lesion. It should be stressed that in BU samples, intramacrophage bacilli were not seen as often as in mice footpads infected with *M. ulcerans*, since the mouse footpad model allows a histopathological evaluation of the complete footpad, whereas in human samples the lesion is usually too extensive to be included in a single biopsy. Consequently, due to the extensiveness of the human lesions, inflammatory infiltrates and AFB were not always represented in the same tissue sample, even when different sections of the same sample were analysed. Altogether, these results direct us towards the re-evaluation of the interactions between *M. ulceran*s and the host phagocytes in the more controlled model of bone marrow-derived macrophages (BMDM) infection.

We quantitatively showed that, like the intracellular mycobacteria *M. tuberculosis* and *Mycobacterium bovis* BCG, *M. ulcerans* is efficiently phagocytosed by BMDM at a 1:1 (bacilli/macrophage ratio) multiplicity of infection (MOI). In this in vitro model of infection, as shown by electron microscopy, *M. ulcerans* was found surrounded by the macrophage phagosomal membrane. In the mouse footpad model of infection, *M. ulcerans* was also found to be extensively phagocytosed by macrophages and neutrophils, in accordance with previous data from our own group (58) and others (16).

The inhibition of *M. ulcerans* phagocytosis and the lack of intramacrophage growth previously described by other authors may be explained by the use of high MOIs (10:1) (3, 70), resulting in early mycolactone-dependent damage of macrophages. In support of our interpretation, it is known that at high MOIs other pathogenic mycobacteria also show cytotoxic activity against cultured cells. Ramakrishnan and colleagues (69) showed that, when macrophages of the J774A.1 cell line were infected with *M. marinum* at an MOI of 1:1 or less, no cytopathic effect was observed in the macrophage's monolayers. However, at higher MOIs, macrophage cell death was detected by the piknotic appearance of cell nuclei (69). Similar results were found by other authors in infections by *M. tuberculosis* (31, 49).

However, the demonstration of *M. ulcerans* inside phagocytes in vivo and of the capacity of macrophages to phagocytose this pathogen in vitro is not conclusive of *M. ulcerans* being an intracellular pathogen. Using the in vitro model of BMDM infection, we showed for the first time that after phagocytosis, *M. ulcerans* grows inside macrophages, as is the case of other pathogenic mycobacteria. The period of intramacrophage growth was dependent on the cytotoxicity of the strain. Low cytotoxic strains grew within macrophages for extended periods of time, whereas highly cytotoxic strains grew within macrophages for shorter periods of time, killing the macrophage after a period on intracellular multiplication due to the activity of mycolactone, becoming thereafter extracellular.

One of the characteristics of intracellular pathogens is their relative low cytotoxicity (4). In fact, it would be intuitive to expect intracellular pathogens to have low direct cytotoxicity, so that no damage would be imparted to the host cells. However, it is becoming progressively evident that a fundamental step in the pathogenic cycle of intracellular pathogens is their ability to lyse and egress from the host cell after termination of intracellular replication, to infect other cells within the same host, or to be transmitted to a new susceptible host (4). In fact, the production of toxins that directly lyses host's cells is a mechanism commonly used by extracellular pathogens, but not uncommon among intracellular pathogens (87). Different intracellular pathogens induce cell death in the host cell to subvert normal host defense responses, mainly by producing toxins that express their enzymatic activity in the cell cytosol (87). *Shigella* Spp are known to induce apoptosis of the host cell, both in vitro and in vivo (90, 91). These species cause a diarrheal disease with a variable clinical picture and the killing of the host cell is a critical point, so the

bacteria can be transmitted through fecal/oral route (87). The *Shigella* genes responsible for invasion and cytotoxicity are located in a plasmid that encodes a secretion type-III apparatus and the toxins. Escape from the phagosome and induction of apoptosis is dependent on the expression and secretion of the plasmid antigens (8, 9). Unlike *Shigella* Spp, *Salmonella typhimurium* is able to grow inside macrophage's phagolysosome (2, 7, 67) but also induces macrophage apoptosis through a type-III secretion mechanism (54). In the case of *Legionella pneumophila*, upon termination of its intracellular replication, which is the hallmark of Legionnaires' disease, the pathogen induces cytolysis of infected macrophages to allow egress of intracellular bacteria. This process of cytolysis is induced by a pore-forming toxin and it was shown to play a major role in pulmonary immunopathology in vivo (4).

The above observations show that several intracellular pathogens reconcile cytotoxicity with intracellular parasitism, inducing cell death after a period of intracellular multiplication to spread to different cells in the same host or to a new host.

Although in some cases, killing of the host cell is detrimental to the host response, apoptosis of macrophages infected with other infectious agents, mainly intracellular pathogens, may be beneficial to the host. In fact, apoptosis of human monocytes has been shown to limit *M. bovis* BCG and *Mycobacterium avium* growth (28, 53). In addition, low virulent strains of *M. tuberculosis* induce high levels of macrophage apoptosis when compared with highly virulent strains (68).

The machinery required to mycolactone production is encoded in a giant plasmid (80). It is still not known if and how mycolactone production is regulated by *M. ulcerans*. In this sense, if the expression of the polyketide synthases required to produce mycolactone are subjected to an orchestrated pattern of transcriptional control during the initial phase of intracellular growth, that would allow the pathogen a period of growth inside the host cell. After reaching a significant intracellular bacterial load, mycolactone synthesis would be activated, resulting in lysis of the host cell. Our data showing that *M. ulcerans* infection of the J774A.1 macrophage's cell line at different MOIs induces cell death when a similar bacterial load is achieved, although at different time points, is in accordance with such a putative mechanism of switching off mycolactone production. As discussed above, this kind of mechanism is used by other pathogenic bacteria including *L. pneumophila* (4).

Another argument in favor of *M. ulcerans* being an intracellular pathogen is the presence in this pathogen of genes known to be important for the intracellular multiplication of other pathogenic mycobacteria, such as *M. tuberculosis*. It has been shown that during periods of survival within macrophages, *M. tuberculosis* specifically express several genes and that some of them are essential for its survival. Namely, genes of the *fadE28* gene cluster appear to be critical for *M. tuberculosis* to survive inside macrophages (72). These genes have been proposed to be involved in lipid transport and degradation, and its likely function consists in assimilating exogenous lipids from the host cell membranes (72). Additionally, genes of the *lpqY* system were shown to be important for survival in nonactivated macrophages and are also important for *M. tuberculosis* growth in vivo. Also *PstA1* and *pstC2*, members of a putative operon, are each essential for *M. tuberculosis* to grow in macrophages (72). *PhoP/ PhoQ*, are known to control transcription of key virulence genes essential for survival in host cells in diverse intracellular bacterial pathogens, including *Salmonella* sp., *Shigella* sp., *Yersinia* sp. and *M. tuberculosis* (62). *Erp* codes for a surface-exposed protein required for multiplication and intracellular growth (13). The genes referred above are also present in the genome of *M. ulcerans*, showing that this species has genes required for intracellular growth.

With the publication of the complete *M. ulcerans* genome sequence (81), a comprehensive search for other *M. ulcerans* genes required to intracellular growth will be important to better understand the biology of the infection caused by this pathogen.

Our observation, in experimental mouse infections and in BU biopsies, of progressive enlargement of the clumps of extracellular AFB away from the areas of colocalization with inflammatory cells suggests that there is extracellular growth of *M. ulcerans* following the shedding of the bacilli from lysed, infected macrophages. Taking into consideration this observation and the results showing that *M. ulcerans* bacilli (i) are efficiently phagocytosed by mouse macrophages in vivo and in vitro, (ii) are present inside macrophages in mouse footpad and peritoneal infections and in BU tissues, and (iii) grows within cultured macrophages, we propose that the growth of *M. ulcerans* would take place, in a manner similar to that of *M. tuberculosis* (18, 38), by both intracellular and extracellular multiplication in distinct areas of the lesion.

The impact of extracellular *M. ulcerans* in the progression of BU is still not elucidated but our results suggest that intramacrophage *M. ulcerans* has an impact in the progression of the disease, as it will be discussed below.

Mycolactone-mediated inhibition of tumor necrosis factor production by *M. ulcerans*-infected macrophages. Tumor necrosis factor (TNF) is a key cytokine in the activation of the macrophage's effector mechanisms (25). However, its role and relevance in *M. ulcerans* infections was not yet evaluated. Moreover, it has been shown that mycolactone inhibits TNF production by phorbol myristate acetate-ionomycin-activated T-cell lines and lypopolysacharide (LPS)-activated human monocytes (59). As previously discussed, *M. ulcerans* induces a cellular inflammatory response in specific areas of the infectious lesion, with the recruitment of macrophages, both in BU patients (39; chapter 2) and in experimental models of infection (58). These results suggest that *M. ulcerans* induces the production of cytokines and chemokines responsible for the recruitment and, possibly, activation of immune cells.

In chapter 3, we first characterized the TNF production by BMDM infected with different strains of *M. ulcerans* and showed that BMDM produce different amounts of TNF, depending on the cytotoxicity/virulence of the strain. Indeed, macrophages infected with highly cytotoxic/virulent strains produce low amounts of TNF, whereas macrophages infected with low cytotoxic/virulent strains produce higher amounts of TNF. The low production of TNF by macrophages infected with mycolactone-producing *M. ulcerans* was not due to an early death of macrophages, since, even at low MOIs, for which no cytotoxic effect was seen, there was no relevant production of this cytokine. Moreover, macrophages infected with the same virulent *M. ulcerans* produced significant amounts of another cytokine (see below). The decreased production of TNF observed in macrophages infected with highly virulent mycolactone-producing *M. ulcerans* strains was shown to be mycolactone dependent. Indeed, macrophages infected with a mycolactone-negative mutant strain of *M. ulcerans* produced high amounts of TNF, and this production was inhibited by the addition of mycolactone.

Inhibition of TNF by other pathogenic mycobacteria has been shown in the past (24, 30, 75). In fact, it is known that strains of *M. avium* that proliferate extensively in cultured macrophages are able to delay the TNF production by the host cell, when compared with strains

with lower capacity to grow in these cells (30). In accordance, the same authors showed that addition of TNF in the beginning of the infection leads to the inhibition of the proliferation of strains that grow in macrophages, with no significant effect for strains with reduced capacity to grow in these cells (30). Likewise, the virulent reference strain *M. tuberculosis* H37Rv inhibits the production of TNF by infected macrophages in a higher extent when compared with the nonvirulent strain H37Ra (24). It is possible that this inhibition may be a strategy used by mycobacteria to prevent macrophage activation, allowing for an extended period of intracellular residence and multiplication. The production of mycolactone by intramacrophage *M. ulcerans*, and the consequent inhibition of TNF production by macrophages, may be a similar mechanism used by *M. ulcerans* to allow an extended intracellular growth in the host, rendering macrophages unable to control the proliferation of mycolactone-producing strains. Supporting this hypothesis, we have shown that TNF p55 deficient-mice are more susceptible to infection by low and intermediate virulent strains of *M. ulcerans*, but not to the infection by a highly virulent strain that induces the production of negligible amounts of TNF in infected macrophages.

TNF was shown to have important roles in mycobacterial infections (78) including, among others: (i) orchestration of leukocyte recruitment and development of the granuloma; (ii) activation of effector functions of macrophages, such as RNI and ROI production (discussed bellow); (iii) maturation of dendritic cells (DC).

The in vivo role of TNF in protective responses to mycobacterial infections, such as *M. tuberculosis* and *M. avium* infections, has been assessed through neutralization with anti-TNF antibodies as well as using TNF p55 deficient-mice (27, 46). Both approaches resulted in the decreased development of granulomas, failure to restrict mycobacterial growth, and decreased survival of infected mice. In *M. ulcerans* infection, the role of TNF is still not completely understood. However, due to its important role in the organization and maintenance of the granuloma, which has been shown to be a protective structure in infections by intracellular mycobacteria (6, 27, 32, 46), it is possible that TNF plays an important role in the organization and maintenance of this histological structure. In BU, granulomatous histopathology has been indeed associated with the healing of lesions (1, 40, 41, 79, 86). On the other hand, disseminated disease and osteomyelitis have been associated with defects in granuloma formation (42, 79). Accordingly, a correlation between the level of TNF expression and the formation of granulomata has been found in BU lesions (64), supporting our conclusion that TNF

plays a protective role in experimental BU. However, more studies are required to further elucidate this point.

Another important observation in our study was the fact that BMDM infected at low MOIs with mycolactone-producing *M. ulcerans* produce levels of macrophage chemoattractant protein-2 (MIP-2) similar to those produced by BMDM infected with mycolactone-negative mutant strains. MIP-2, being the murine homologue of human interleukin (IL)-8 (83), plays a central role in the recruitment of neutrophils in mice (29, 89). The expression of this chemokine was already reported in lesions of patients infected with *M. ulcerans* (64). Recently, Coutanceau and colleagues showed that a mycolactone-producing strain of *M. ulcerans* induces the expression of MIP-2 in infected macrophages (16). The expression of this chemokine in BU lesions, as well as its production by macrophages in vitro, is in accordance with the results presented by Guarner and colleagues (39) and the results from our laboratory (58), showing that *M. ulcerans* induces inflammatory responses containing neutrophils.

Recently (15), it was shown that purified mycolactone blocks the emigration of DC from the skin to the draining lymph nodes as well as its maturation. Additionally, these authors also showed that DC treated with noncytotoxic concentrations of mycolactone were unable to activate allogeneic T cells. Surprisingly, these effects were not related with TNF, since mycolactone treated, LPS-stimulated DCs were able to produce TNF (15). It is well known that TNF is important for triggering the maturation programme in DCs, inducing DC migration and the upregulation of the molecular pattern required for the activation of T cells (52). Although interesting, the real impact of these results in infections by *M. ulcerans* requires further investigation. In this context, the diffusion of mycolactone in the tissue, the different types of mycolactones as well as the amount of mycolactones produced by different strains of *M. ulcerans* may be factors to take into consideration. In fact, in chapter 3 we showed that mycolactone A/B producing strains were more virulent for mice and more cytotoxic for BMDM when compared with mycolactone C producing strains. These results are in accordance with previous data showing that purified mycolactone A/B is more cytotoxic than mycolactone C (56).

Although it is clear from our data that the outcome of the infection by *M. ulcerans* depends on the type of mycolactone produced, the existence of other factors, such as regulation of mycolactone production, amount of mycolactone produced or, even, other virulence factors, can not be ruled out.

Although, in vitro, mycolactone was shown to inhibit TNF production, the expression of this cytokine, has referred above, has been detected in BU lesion samples (64, 66). It is still not clear which cells produce TNF in BU lesions. It can be hypothesized that TNF may be produced by: (i) infected macrophages when the *M. ulcerans* genes required to produce mycolactone would be switched off; (ii) newly recruited macrophages or other cell types in areas not affected by the toxin; (iii) macrophages or other cell types activated by inflammatory molecules derived from the process of tissue necrosis, regardless the contact with *M. ulcerans* or *M. ulcerans*-derived molecules. It is also not clear if TNF detected in BU lesions has any impact in the control of *M. ulcerans* proliferation. The dose-dependent inhibitory effect of mycolactone on the in vitro production of TNF, as shown by us, suggests that this cytokine may have increased biological relevance in areas of the lesion where mycolactone concentration is low. In fact, it is known that BU lesions can heal spontaneously (73), but the role of TNF and other inflammatory cytokines in this process is currently not known. However, the lack of available methodologies, at the present time, to detect mycolactone in host tissues makes it impossible to clarify this hypothesis. The development of such methodologies will be essential to clarify the role of this and other cytokines in in vivo infections by *M. ulcerans*.

Role of interferon-gamma on the activation of the effector mechanisms of macrophages infected with *M. ulcerans*. Interferon-gamma (IFN-γ), in association with TNF, is a key cytokine for the activation of macrophage's effector functions. To date, IFN-γ-deficient mice are the most susceptible to *M. tuberculosis* infection (12, 26). In these mice, macrophage activation has been shown to be defective, and production of intermediate radicals, such as nitric oxide (NO), is low. These are probably the factors that contribute to the high susceptibility of IFN-γ-deficient mice to *M. tuberculosis* infection (17, 26). However, the existence of other IFN-γ-dependent, NO production-independent, anti-mycobacterial mechanisms can not be ruled out (25).

In human infections by *M. ulcerans*, production of IFN-γ has been suggested to be protective. However, until now, there are no reports showing a direct evidence for the protective role of this cytokine. Therefore, we used the previously developed footpad mouse model of *M. ulcerans* infection, as well as the in vitro infection of BMDM, to study the biological role of IFN-γ in *M. ulcerans* infections, and to clarify the macrophage's effector mechanisms activated upon

infection with different strains of *M. ulcerans*. As compared to wild-type (WT) counterparts, we found that IFN-γ-deficient mice are more susceptible to sub-cutaneous infection by the mycolactone-negative strain 5114, and slightly more susceptible to the intermediate virulent mycolactone-producing *M. ulcerans* 94-1327. For the tested infection doses, no differences were found in the proliferation and in the levels of pathology induced by the highly virulent strain *M. ulcerans* 98-912.

In vitro, at a 1:1 MOI, BMDM activated with IFN-γ controlled the proliferation of *M. ulcerans* 5114 but not of *M. ulcerans* 94-1327 or 98-912. Interestingly, at lower MOIs we found that IFN-γ-activated macrophages controlled the proliferation of the intermediate virulent strain *M. ulcerans* 94-1327.

Macrophage's effector functions were shown to be NO-dependent, since the competitive inhibition of NOS rendered IFN-γ-activated macrophages unable to control *M. ulcerans*. However, mycolactone-producing strains, including *M. ulcerans* 94-1327, did not trigger the production of NO by macrophages. Interestingly, *LRG-47* was shown not to be inhibited significantly by *M. ulcerans* 94-1327, which can contribute to the control of proliferation found at low MOIs. On the other hand, a complete inhibition of *LRG-47* expression was found in macrophages infected with *M. ulcerans* 98-912.

As previously mentioned, different studies have suggested that IFN-γ is associated with protection in human *M. ulcerans* infections (35, 36, 63, 64). All the data published until now are based on the expression of this cytokine in BU lesion samples (64, 66) or on its production by peripheral blood mononuclear cells (PBMC) after stimulation with *M. ulcerans*, *M. ulcerans* antigens or *M. bovis* BCG (35, 36, 63, 66).

The first studies suggesting a role for IFN-γ in the protection against *M. ulcerans* infections were performed in Australian patients with a history of past BU and showed that PBMC from patients, but not from healthy contacts, display a low capacity to produce IFN-γ after in vitro stimulation with live *M. ulcerans* bacilli or *M. bovis* BCG (35, 36). Since household contacts of BU patients showed antibodies against *M. ulcerans*, suggesting a previous contact with this pathogen without any signs of active disease, those authors interpreted this observation as indicating that, in an environment where *M. ulcerans* is endemic, subjects who have not developed clinical disease have been exposed to *M. ulcerans* and developed a protective immune response mediated by IFN-γ (36). In accordance, it has been suggested that a significant

proportion of the population residing in areas of *M. ulcerans* endemicity may be exposed to *M. ulcerans* but do not develop disease (19). Interestingly, Gooding and colleagues found that PBMC from the majority of individuals in the affected group express IL-10, suggesting that this immunosuppressive cytokine was the responsible for the reduced expression of IFN-γ observed in BU patients. Accordingly, Prevot and colleagues (66) showed, in a different study, that the production of IL-10 by PBMC stimulated with killed *M. ulcerans* or *M. bovis* BCG was higher in PBMC from BU patients than from controls. Some of the limitations of these studies with PBMC is the long time of stimulation (6 and 7 days) and the low number of patients involved. It is also possible that the cytokines produced were not *M. ulcerans*-specific, due to the long time of stimulation. In a larger study in Ghana, using overnight stimulation of PBMC with *M. ulcerans* sonicate, it was found that the IFN-γ levels in patients with ulcers were similar to those in patients with healed BU, but higher than those in patients with nodules (63). IL-10 production was found to be higher in patients with active *M. ulcerans* disease than in those with healed lesions, being the pattern of response similar to that seen in tuberculosis (88). An interesting observation by these authors was the wide variation in IFN-γ and IL-10 production among patients which, in part, explains the discrepancies found in Gooding and Prevot's studies that used a lower number of patients.

When PCR analyses was used to quantify the cytokine expression in nodular or ulcerative BU lesions, Prevot and colleagues (66) found a higher expression of IFN-γ and lower expression of IL-10 in the less severe nodular forms, in contrast with lower expression of IFN-γ and higher expression of IL-10 in the ulcerative lesions (66), suggesting that there was suppression of IFN-γ and increased IL-10 production in longer established lesions, as previously suggested by studies of PBMC stimulation (36). However, in a more recent study, using a high number of BU samples, Phillips and colleagues found a median expression of IFN-γ lower in nodules, but in some cases the production of this cytokine was above the median, when compared with the group of ulcerative lesions, showing a high variability in IFN-γ expression among patients (63). The suggestion of Prevot and colleagues (66) that IL-10 was responsible for the low IFN-γ responses in BU patients was not confirmed by Phillips and colleagues (63). In fact, these authors found that patients with the nodular forms of the disease did show low IFN-γ responses in the presence of higher IL-10 responses, but patients with ulcerative disease had the highest IL-10 responses

and, at the same time, showed high IFN-γ expression (63). In fact, these authors did not find any inverse correlation between IFN-γ and IL-10 (63).

As clearly stems from the reports discussed above, although different studies have been done to clarify the nature of the protective immune response to *M. ulcerans* infections, the results are not conclusive and, in some cases, are clearly contradictory. In that sense, the use of the mouse model will be essential to disclose the role of different cytokines, due to the high number of available gene-deficient strains and immunological reagents. In addition to the aspects discussed above, the discrepancies reported by the different authors could be explained by the fact that these studies were performed in different geographical regions of the world. It is known that different isolates of *M. ulcerans* show small differences in their 16s RNA (65), as well as in the types of mycolactones they produce (56). Therefore, differences in virulence of the isolates may also lie at the basis of the discordant results. However, from these studies we know that a high number of patients have to be used in order to obtain accurate results, and, in future studies, it will be important to determine the virulence of the isolate, or isolates, infecting each patient, their cytotoxicity and profile of mycolactones produced. These results may be important for the identification of other *M. ulcerans'* putative virulence factors.

FINAL REMARKS

In the present dissertation we showed for the first time that *M. ulcerans* has a phase of intracellular residence and multiplication in the host. Additionally, we showed that *M. ulcerans* induces TNF production from infected macrophages, being this cytokine essential to control *M. ulcerans* proliferation in vivo. However, mycolactone-producing *M. ulcerans* strains inhibit TNF production from infected macrophages, which leads to an ineffective control of the infection in vivo. We also showed that mycolactone-producing strains are able to inhibit several IFN-γ-dependent microbicidal mechanisms of macrophages.

The findings here presented have important consequences for the host's resistance to the infection. Additionally, the intramacrophage growth phase of *M. ulcerans,* as well as the role of the inflammatory cytokines TNF and IFN-γ, has to be taken in consideration for the future development of a specific vaccine or new drug therapies against BU.

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