Mycobacterium ulcerans Triggers T-Cell Immunity followed by Local and Regional but Not Systemic Immunosuppression

Alexandra G. Fraga, Andrea Cruz, Teresa G. Martins, Egídio Torrado, Margarida Saraiva, Daniela R. Pereira, Wayne M. Meyers, Françoise Portaels, Manuel T. Silva, António G. Castro and Jorge Pedrosa

Infect. Immun. **2011, 79(1):421. DOI: 10.1128/IAI.00820-10. Published Ahead of Print 25 October 2010.**

INFECTION AND IMMUNITY, Jan. 2011, p. 421–430 Vol. 79, No. 1
0019-9567/11/$12.00 doi:10.1128/IAI.00820-10

Mycobacterium ulcerans Triggers T-Cell Immunity followed by Local and Regional but Not Systemic Immunosuppression▽

Alexandra G. Fraga,[1] Andrea Cruz,[1] Teresa G. Martins,[1] Egídio Torrado,[1]‡ Margarida Saraiva,[1] Daniela R. Pereira,[1] Wayne M. Meyers,[2] Françoise Portaels,[3] Manuel T. Silva,[4] 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]; Armed Forces Institute of Pathology, Washington, DC[2]; Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium[3]; and Institute for Molecular and Cell Biology, Porto, Portugal[4]

Received 29 July 2010/Returned for modification 7 September 2010/Accepted 17 October 2010

Buruli ulcer is a neglected infectious disease caused by *Mycobacterium ulcerans* and is characterized by necrotic cutaneous lesions induced by the exotoxin mycolactone. Despite evidence of Th1-mediated protective immunity, *M. ulcerans* infection has been associated with systemic immunosuppression. We show that early during mouse infection with either mycolactone-positive or negative strains, pathogen-specific gamma interferon (IFN-γ)-producing T cells developed in the draining lymph node (DLN). $CD4^+$ cells migrated to the infection foci, but progressive infection with virulent *M. ulcerans* led to the local depletion of recruited cells. Moreover, dissemination of virulent *M. ulcerans* to the DLN was accompanied by extensive DLN apoptotic cytopathology, leading to depletion of $CD4^+$ T cells and abrogation of IFN-γ expression. Advanced footpad infection with virulent *M. ulcerans* did not induce increased susceptibility to systemic coinfection by *Listeria monocytogenes*. These results show that infection with *M. ulcerans* efficiently triggers a mycobacterium-specific T-cell response in the DLN and that progression of infection with highly virulent *M. ulcerans* leads to a local and regional suppression of that immune response, but without induction of systemic immunosuppression. These results suggest that prophylactic and/or therapeutic interventions to prevent dissemination of *M. ulcerans* to DLN during the early phase of infection would contribute for the maintenance of protective immunity and disease control.

Buruli ulcer (BU) is an emerging neglected tropical disease caused by *Mycobacterium ulcerans* and is characterized by nonulcerative lesions that can evolve into severe ulcers (41, 70).

Infection by *M. ulcerans* poses a unique challenge for the host immune system due to the secretion of the highly cytotoxic lipidic exotoxin mycolactone (17). Mycolactone has been suggested to suppress the development of local and systemic immune responses by inhibiting cytokine production during active BU (19–21, 42, 72, 75) and compromising T-cell priming by suppressing dendritic cell function (11). However, there are no studies on the association of BU with opportunistic infections, suggesting that the immunosuppressive effects of mycolactone might not be systemic.

The observations that active BU occurs in a small proportion of exposed individuals (14, 58) and when it develops frequently heals spontaneously (69) suggest the existence of protective immunity. Although the protective mechanisms remain largely unknown, several studies support that adaptive cell-mediated immunity (CMI) is relevant for the control of *M. ulcerans* (reviewed in reference 53). In fact, (i) *M. ulcerans* has an intramacrophage growth phase (66), (ii) resistance to BU is associated with the development of T helper (Th) 1 type responses and granulomata (10, 20, 21, 23, 42, 48, 63, 70, 72), (iii) the positivity of the delayed-type hypersensitivity (DTH) burulin test increases from early to advanced phases (15, 31), (iv) the histopathology of healing BU lesions in patients submitted to antibiotic treatment is consistent with CMI (49), (v) *Mycobacterium bovis* BCG vaccination induces transient protection in humans and in experimental infections (16, 55, 60, 62, 68), (vi) infection with HIV increases the risk of developing BU and more aggressive multifocal forms (27, 64), and (vii) the pattern of cytokine expression in BU lesions conforms with CMI and DTH (28, 38, 67).

Therefore, to clarify whether the *M. ulcerans* infectious process interferes with the development of protective immunity and whether systemic immunosuppression is induced, we monitored the host immune response in the mouse model, not only in the primary site of infection but also in the draining lymph node (DLN), where the initiation of the adaptive immunity takes place.

MATERIALS AND METHODS

Animals. Eight-week-old female wild-type, nude, and Rag2-deficient mice in a BALB/c background were obtained from Charles River (Barcelona, Spain). Rag-deficient BALB/c mice transgenic for the DO11.10 α/β T-cell receptor (TCR) were from Anne O'Garra (NIMR, London, United Kingdom). The studies involving animals were approved by the review committees of ICVS and the Portuguese Governmental Agency Direcção Geral de Veterinária.

Experimental infections. The different *M. ulcerans* strains used in the present study were selected based on their virulence for mice (35, 65) and on the type of mycolactone produced (26, 32, 59). *M. ulcerans* 5114 is a low-virulence Mexican

* Corresponding author. Mailing address: Life and Health Sciences Research Institute, School of Health Sciences, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal. Phone: 351 253604870. Fax: 351 253604809. E-mail: jpedrosa@ecsaude.uminho.pt.

† A.G.C. and J.P. contributed equally to this study.

‡ Present address: Trudeau Institute, Saranac Lake, NY 12983.

▽ Published ahead of print on 25 October 2010.

isolate that does not produce mycolactone (32) due to the loss of key genes involved in the synthesis of this macrolide (59). The Australian strain 94-1327 was isolated from an ulcer and produces the mycolactone C (32). Strain 97-1116 was isolated in Benin from a patient presenting with a plaque and was found to produce mycolactones A and B (32); *M. ulcerans* 98-912 was isolated in China from an ulcer and produces mycolactone D (26). All *M. ulcerans* strains used in the present study are from the collection of the Institute of Tropical Medicine (ITM), Antwerp, Belgium. The isolates were grown on solid Middlebrook 7H9 medium at 32°C for approximately 1 month.

For the preparation of *M. ulcerans* inoculum, bacteria were diluted in phosphate-buffered saline (PBS) and vortexed using glass beads. The number of acid-fast bacilli (AFB) in inocula was determined by using Ziehl-Neelsen staining (Merck) according to the method described by Shepard and McRae (50). For *L. monocytogenes* strain EGD inocula, bacteria were grown in brain heart infusion broth at 37°C to mid-log phase and frozen in aliquots at −80°C. Mice were either infected subcutaneously in the footpad with *M. ulcerans* (3×10^5 AFB) alone or coinfected intravenously with *L. monocytogenes* (5×10^3 CFU).

Bacterial proliferation in footpad. *M. ulcerans* proliferation was evaluated in footpad tissues of infected mice. Briefly, tissue specimens were minced, resuspended in PBS containing 0.04% Tween 80 (Sigma), and vortexed with glass beads. The number of AFB in the bacterial suspension was determined by using the method of Shepard and McRae (50).

Flow cytometry. Single cell suspensions of the popliteal lymph nodes were stained with a combinations of fluorochrome-labeled monoclonal antibodies specific for CD3 (clone 145-2C11), CD19 (clone 1D3), CD4 (clone RM4-5), CD8 (clone 53-6.7), CD62L (clone MEL-14), BrdU (clone PRB-1), and transgenic DO11.10 TCR (KJ1-26). Cells were analyzed by using CellQuest software on a Becton Dickinson FACSCalibur flow cytometer.

Cell suspensions from infected footpads. Infected footpads were collected and incubated in incomplete Dulbecco modified Eagle medium (DMEM; Gibco) and 125 U collagenase XI (Sigma-Aldrich)/ml, for 2 h at 37°C. Each footpad was filtered through a 40-μm-pore-size nylon cell strainer and washed with DMEM supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich).

Real-time PCR. Cell suspensions from the DLN and the infected footpad were frozen in TRIzol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's protocol. Quantification of message for ubiquitin and gamma interferon (IFN-γ) was carried out with Sybr green (Qiagen). The sequences for the ubiquitin and the IFN-γ reaction were designed and synthesized by TIB MolBiol and were as follows: ubiquitin forward, GCTGGTGAAAAGGACC TCT; ubiquitin reverse, CACAGGACTAGAACACCTGC; IFN-γ forward, TG GCAAAAGGATGGTGACATG; and IFN-γ reverse, GACTCCTTTTCCGCT TCCTGA. The primers and probes for Cxcl9, Cxcl10, and Cxcl11 were purchased from Applied Biosystems gene expression assays.

ELISPOT assay. As previously described, the enzyme-linked immunospot (ELISPOT) assay was used for the detection of antigen-specific IFN-γ-producing cells from infected DLN (13). Cells were stimulated with either 10 μg of peptide Ag85A/ml or 10 μg of tuberculin PPD derivative of *M. tuberculosis* (Statens Seruminstitut)/ml. The cells cultured in the absence of antigen or cells from uninfected mice did not produce detectable spots.

Adoptive transfer. The adoptive transfer was performed as previously described (7). Briefly, splenocytes from DO11.10 TCR transgenic mice (10^6 transgenic $CD4^+$ T cells) were injected intravenously into uninfected mice or mice that were infected for 1 week with *M. ulcerans*. Mice were primed 2 days after adoptive transfer with 3 mg of ovalbumin (OVA; Sigma-Aldrich) subcutaneously in the *M. ulcerans*-infected footpad.

T-cell turnover by BrdU incorporation. Bromodeoxyuridine (BrdU; 1 mg; Sigma) was injected intraperitoneally into mice, twice a day, for 3 days prior analysis. Cells were harvested from the DLN, labeled with anti-CD4 and anti-KJ1.26, washed with PBS, and fixed overnight at 4°C with 1% paraformaldehyde–0.2% Tween 20. Cells were incubated with 50 U of DNase (Roche)/ml at 37°C for 1 h, washed with Coffman's balanced salt solution–1% bovine serum albumin–0.2% Tween 20, and stained with anti-BrdU (eBioscience) or fluorescein isothiocyanate isotype control for 45 min at 4°C before flow cytometric analysis.

Bacterial load determination. The DLN, spleens, and livers were aseptically excised and individually homogenized. Serial dilutions of the organ homogenate were plated on nutrient 7H9 agar or brain heart infusion agar medium (Pronadisa). *M. ulcerans* CFU was counted after 6 to 8 weeks of incubation at 32°C, and the *L. monocytogenes* CFU were counted after 24 h of incubation at 37°C.

Histological and immunofluorescence studies. DLN and footpads were harvested, fixed in buffered formalin, and embedded in paraffin. Light-microscopy studies were performed on tissue sections stained with hematoxylin and eosin (H&E). For immunofluorescence staining, antigen retrieval was performed with citrate buffer (Lab Vision Corp.) for 30 min. Apoptotic cells were stained with anti-human/mouse caspase-3 active (R&D) at 1:250 for 3 h at room temperature and then incubated with Alexa Fluor 568 (Molecular Probes) for 1 h at room temperature. For the detection of $CD4^+$ cells, the sections were incubated with phycoerythrin-conjugated anti-CD4 (clone RM4-5; Santa Cruz Biotechnology) at 1:50 overnight at 4°C. DAPI (4′,6′-diamidino-2-phenylindole) was used to detect nuclei. Images were obtained with an Olympus BX61 microscope.

Statistical analysis. Differences between the means of experimental groups were analyzed with the two-tailed Student *t* test. Differences with a *P* value of ≤0.05 were considered significant.

RESULTS

T cells are required to control infection by low-virulence *M. ulcerans* 5114. To understand the mechanisms underlying the susceptibility of hosts to progressive infections by *M. ulcerans*, we investigated whether T cells are involved in the host response to this intracellular pathogen. For that, we infected mice deficient in the recombinase gene RAG-2 that results in the lack of V(D)J recombination activity, TCR rearrangement, and consequent inability to generate mature lymphocytes (51–52) or nude mice that present a deteriorated or absent thymus, also resulting in the inability to generate mature T lymphocytes (39). During infection with the mycolactone-negative *M. ulcerans* strain 5114, wild-type mice effectively controlled bacterial proliferation in the footpad, whereas $Rag2^{-/-}$ and nude mice showed a 3-$\log_{10}$ increase in bacterial burdens (Fig. 1A and C). In contrast, no differences were found in the proliferation of the highly virulent strain 98-912 (Fig. 1B and D), regardless of the presence of lymphocytes in the host. These data suggest a role for T-cell-mediated responses in the control of infection by a low-virulence *M. ulcerans* strain and in the failure of this response to control infections by a mycolactone-producing strain.

***M. ulcerans* induces an early accumulation of $CD4^+$ cells in the DLN, regardless the strain virulence.** We next evaluated whether the lack of T-cell-dependent protection in mice infected with *M. ulcerans* 98-912 was due to T-cell anergy (19–21) or to mycolactone-mediated impairment of maturation/migration of dendritic cells and, consequently, T-cell activation (11). Infection with strain 5114 induced an initial increase in the total number of DLN cells to a level that was maintained throughout the experimental infection (Fig. 2A). With the mycolactone-producing strain 98-912, we observed a 27-fold increase in the number of DLN cells during the first 2 weeks, followed by a sharp decrease (Fig. 2A). Analysis of the DLN lymphocytic populations in mice infected with either *M. ulcerans* strains revealed that both $CD3^+$ (Fig. 2B) and $CD19^+$ (Fig. 2C) cells followed the cellular dynamics described above, with a predominance of the $CD4^+$ phenotype (Fig. 2D and E).

To investigate whether the host mounts antigen-specific $CD4^+$ T-cell responses in the DLN during a progressive *M. ulcerans* infection, we adoptively transferred OVA-specific TCR transgenic cells ($KJ1.26^+$) to previously infected mice, later challenged with the cognate antigen in the infected footpad. Mice infected with either strain were not compromised in their ability to initiate a T-cell response to OVA in the DLN, since $CD4^+KJ1.26^+$ T-cell accumulation was increased compared to uninfected controls (Fig. 3A). Consistent with the occurrence of T-cell activation, CD62L was downregulated on $CD4^+KJ1.26^+$ cells (Fig. 3B). Both T-cell accumulation and activation were more pronounced in mice previously infected

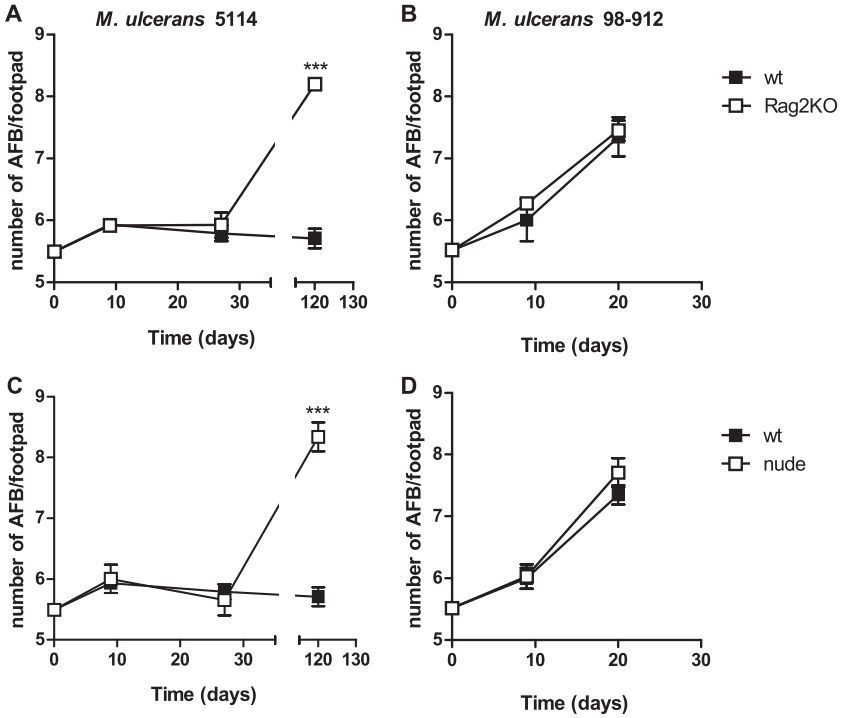

FIG. 1. Lymphocytes are protective against infection with a low-virulence strain but not with a highly virulent strain of *M. ulcerans*. Wild-type (■), Rag2$^{-/-}$ (□), or nude (□) mice were subcutaneously infected in the footpads with 3×10^5 AFB of *M. ulcerans* 5114 (A and C) or 98-912 (B and D). At different time points, the infected footpads were collected and homogenized for bacterial load determination by AFB counting. The data points represent the means ± the standard errors of the mean (SEM) ($n = 5$). The results of one experiment representative of two are shown. The statistical significance was calculated using the Student *t* test, comparing wild-type and immunodeficient mice (***, $P < 0.001$).

with *M. ulcerans* 98-912. Thus, the infectious process at the site of antigen challenge did not compromise the initiation of cellular immune responses. Moreover, the infection did not compromise the proliferative activity of DO11.10 CD4$^+$ cells in the DLN (Fig. 3C). Altogether, these data indicate that early infection by *M. ulcerans* does not compromise the development of antigen-specific T-cell responses in the DLN.

Early during infection, highly virulent *M. ulcerans* induces the development of IFN-γ-producing pathogen-specific T cells in the DLN. Given the onset of cellular immune responses in the DLN, we questioned whether CD4$^+$ T cells were able to mount a mycobacterium-specific Th1 immune response. Correlating with T-cell dynamics (Fig. 2B), we found a moderate increase in the expression of IFN-γ mRNA that was maintained during infection with strain 5114, while infection with strain 98-912 induced a higher peak of IFN-γ expression at day 15, followed by a sharp decline (Fig. 4A). To assess whether the increased expression of IFN-γ was associated to generation of mycobacterium-specific T cells in the DLN, the frequency of IFN-γ-producing T cells responding to the mycobacterial antigens Ag85A (Fig. 4B) or PPD (data not shown) was determined by ELISPOT assay at day 14 postinfection. Infection with either *M. ulcerans* strain elicited a mycobacterium-specific Th1 cell response, with the number of IFN-γ-producing cells being significantly higher in mice infected with the highly virulent strain (Fig. 4B). Thus, we conclude that a transient *M. ulcerans*-specific, IFN-γ-producing T-cell response occurs in the DLN at the early phase of footpad infection, regardless the secretion of mycolactone.

During infection with toxigenic *M. ulcerans*, CD4$^+$ T cells are early recruited to the footpad but later are depleted. Despite the development of specific Th1 responses, *M. ulcerans* 98-912 is not controlled in infected footpads (Fig. 1), raising the possibility of a deficient T-cell recruitment. To address this, we analyzed the dynamics of T-cell migration from the DLN to the primary focus of infection. We therefore determined the expression of CXCL9, CXCL10, and CXCL11 in infected footpads, chemokines associated with trafficking of Th1 cells (36). Infection with either *M. ulcerans* strain induced the expression of these chemokines, with the highly virulent strain triggering a higher expression early during infection (Fig. 5A to C). Preceding the ulceration of footpads infected with strain 98-912, the expression of those chemokines dropped sharply (Fig. 5A to C). These results suggest that the setting for the recruitment of T cells is not impaired early during infection with either strain of *M. ulcerans*.

Alternatively, the lack of protection against the highly virulent strain could be associated with the local destruction of infiltrating lymphocytes (35) due to the cytotoxicity of mycolactone. Therefore, we determined the number of total and CD4$^+$ T cells in footpads. A steady increase in the total cell number was observed throughout the period of infection with strain 5114, but not with 98-912 (Fig. 5D). Infection with the mycolactone-negative strain led to a significant increase in the CD4$^+$ population during the first 15 days (Fig. 5E). CD4$^+$ cells were found at the focus of infection with strain 98-912 but, as infection progressed, a marked decrease was observed (Fig. 5E), correlating with the emergence of ulceration. These data

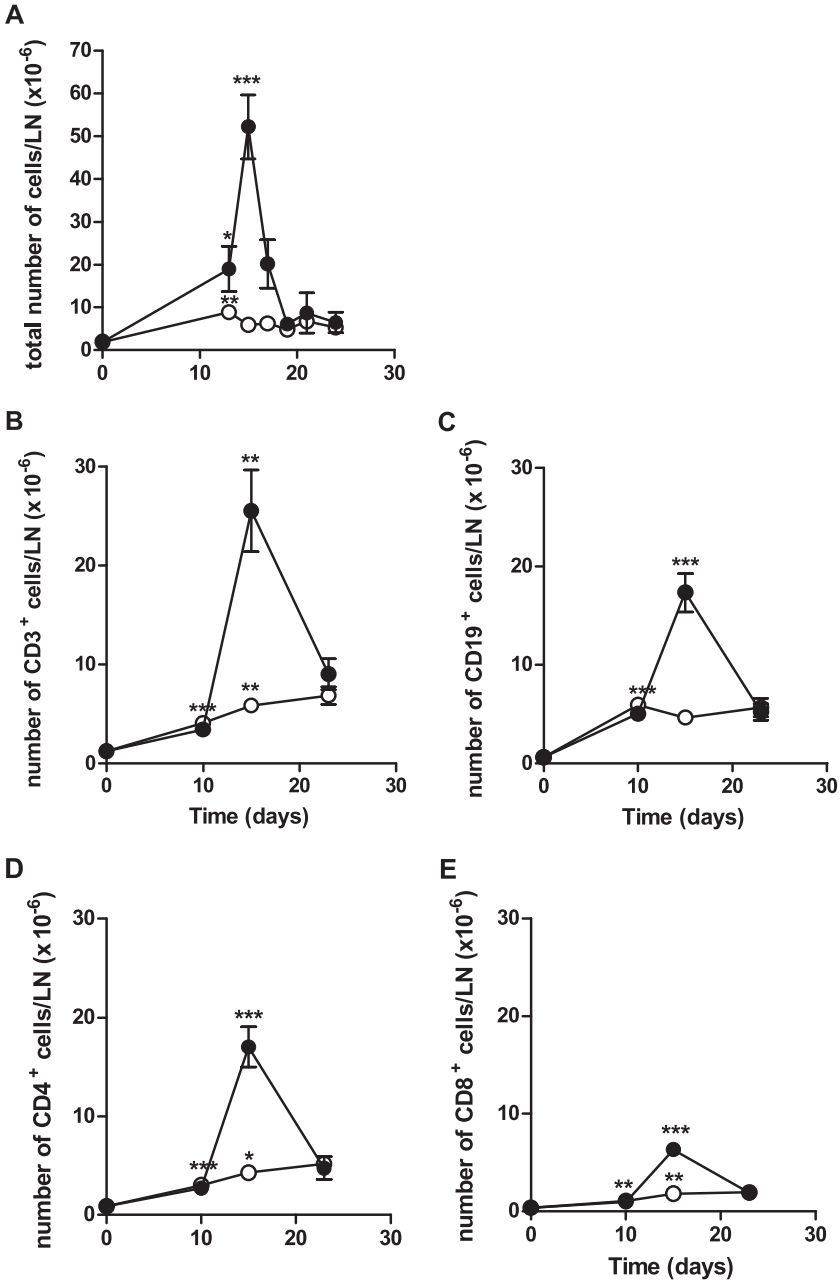

FIG. 2. Infection with *M. ulcerans* induces an early increase in the DLN $CD4^+$ population, followed by a sharp decrease in the case of the highly virulent strain. Mice were subcutaneously infected in the footpad with 3×10^5 AFB of *M. ulcerans* 5114 (○) or 98-912 (●). At different time points postinfection, the total number of leukocytes in the DLN of the infected footpad was determined (A), and the frequencies of $CD3^+$ (B), $CD19^+$ (C), $CD4^+$ (D), and $CD8^+$ (E) cells were determined by flow cytometry. The data points represent the means ± the SEM ($n = 5$) of four independent experiments. The statistical significance was calculated using the Student *t* test, comparing each time point with noninfected mice (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

show that, although the recruitment of DLN-differentiated T cells to the footpad occurs at early time points, the infiltrating cells are later destroyed at the site of infection by the mycolactone-producing strain.

Later during infection by the highly virulent strain of *M. ulcerans*, the DLN are colonized and destroyed by apoptotic cell death. The rapid decrease in the number of T cells in the DLN (Fig. 2B, D, and E) may also contribute to the lack of protection. Supporting this hypothesis, DLN histological analysis (Fig. 6) revealed that progression of infection with *M. ulcerans* 98-912, as well as with the mycolactone-positive strains 94-1327 or 97-1116, was accompanied by severe damage, with extensive areas of apoptotic and necrotic cellular alterations, leading to the destruction of the lymphoid tissue, before the onset of ulceration (Fig. 6G, H, K, L, O, and P). In contrast, no relevant alterations were observed in the structure of the DLN during infection with the mycolactone-negative strain (Fig. 6A to D).

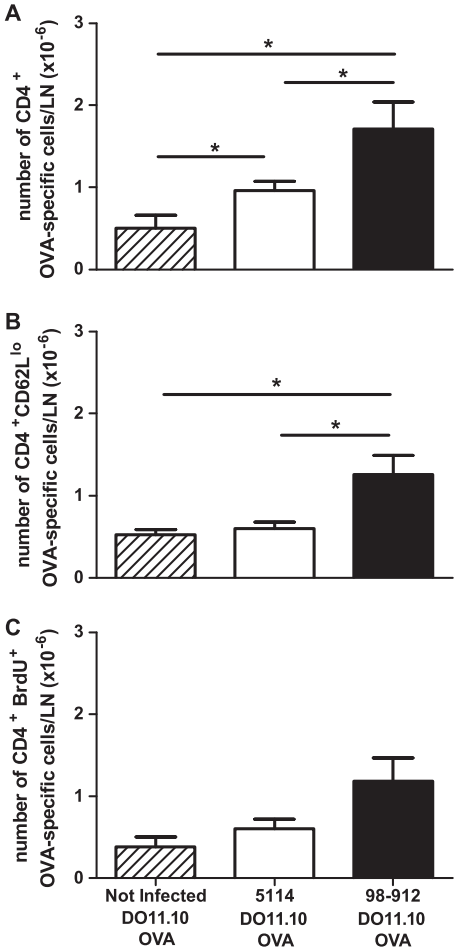

FIG. 3. Early development of antigen-specific $CD4^+$ T-cell responses in the DLN during infection with *M. ulcerans*. OVA-specific TCR transgenic cells ($KJ1.26^+$) from DO11.10 mice were intravenously transferred to noninfected mice (▨) or mice subcutaneously infected for 7 days in the footpad with 3×10^5 AFB of *M. ulcerans* 5114 (□) or 98-912 (■). These mice were challenged subcutaneously 2 days later in the infected footpad with OVA. Three days after cognate antigen challenge, the total number of cells in the DLN was calculated. The frequencies of $CD4^+$ OVA-specific cells (A), $CD4^+$ $CD62L^{low}$OVA-specific cells (B), and $CD4^+$ $BrdU^+$ OVA-specfic cells (C) were determined by flow cytometry. The data points represent means ± the SEM ($n = 5$). The results of one experiment representative of three total experiments are shown. The statistical significance was calculated using the Student *t* test (*, $P < 0.05$).

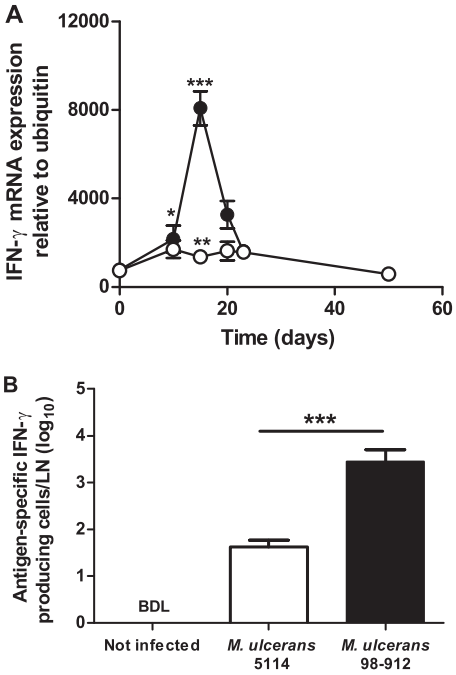

FIG. 4. IFN-γ is produced by antigen-specific T cells in the DLN early during *M. ulcerans* infections. Mice were either not infected or were subcutaneously infected in the footpad with 3×10^5 AFB of *M. ulcerans* 5114 (○ or white bars) or 98-912 (● or black bars). (A) At different times postinfection, mRNA for IFN-γ was determined in the DLN by real-time PCR. The data points represent the means ± the SEM ($n = 5$) for each time point. One representative experiment of four is shown. Statistical significance was calculated using the Student *t* test, comparing each time point with noninfected mice. (B) The number of Ag85-specific IFN-γ-producing T cells was determined in the DLN by ELISPOT assay, 14 days after infection. The data points represent the means ± the SEM ($n = 4$). The results of one experiment representative of two total experiments are shown. Statistical significance was calculated by using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Previous reports showed that mycolactone-mediated cytotoxicity is associated with apoptotic cell death (4, 18, 35) involving caspase-3 activation (56). We therefore determined the number of cells with active caspase-3 in the DLN by day 15 postinfection. We found an increased number of active caspase-3-positive cells in mice infected with strain 98-912, compared to the mycolactone-negative strain (Fig. 6Q). This increase is associated with tissue destruction and was accompanied by a local increase in bacterial burden, in contrast to DLN infected with *M. ulcerans* 5114 (Fig. 6R). These results show that virulent mycolactone-producing strains progressively infect the DLN, inducing massive apoptosis-mediated cell destruction, further compromising a sustained T-cell response.

Infection with the highly virulent strain of *M. ulcerans* does not induce systemic immunosuppression. The occurrence of both local and systemic immunosuppression, in association with a compromised $CD4^+$ T-cell response, has been suggested both in BU patients and in animal models (11, 19–21, 25, 75). After showing that local and regional immunosuppression eventually ensues by depletion of T cells, we addressed the occurrence of systemic immunosuppression. For this, *M. ulcerans*-infected mice were systemically coinfected with a sublethal dose of *L. monocytogenes*, when footpad lesions were already at an advanced stage. *L. monocytogenes* is a facultative intracellular pathogen for which the host requires $CD4^+$ and $CD8^+$ T-cell-mediated immunity for protection (5, 33, 37, 47). In fact, it has been shown that the selective depletion of $CD4^+$ or $CD8^+$ cells or both renders mice more susceptible to a sublethal dose of *L. monocytogenes* (47). In addition, nude or SCID mice, although resistant to *L. monocytogenes* at early time points, ultimately succumb to infection (5, 33). Therefore, this model of coinfection will allow us to determine whether *M. ulcerans* infection induces systemic immunosuppression. Our data show that infection with *M. ulcerans* did not render mice more susceptible to the coinfection, as assessed by bacterial

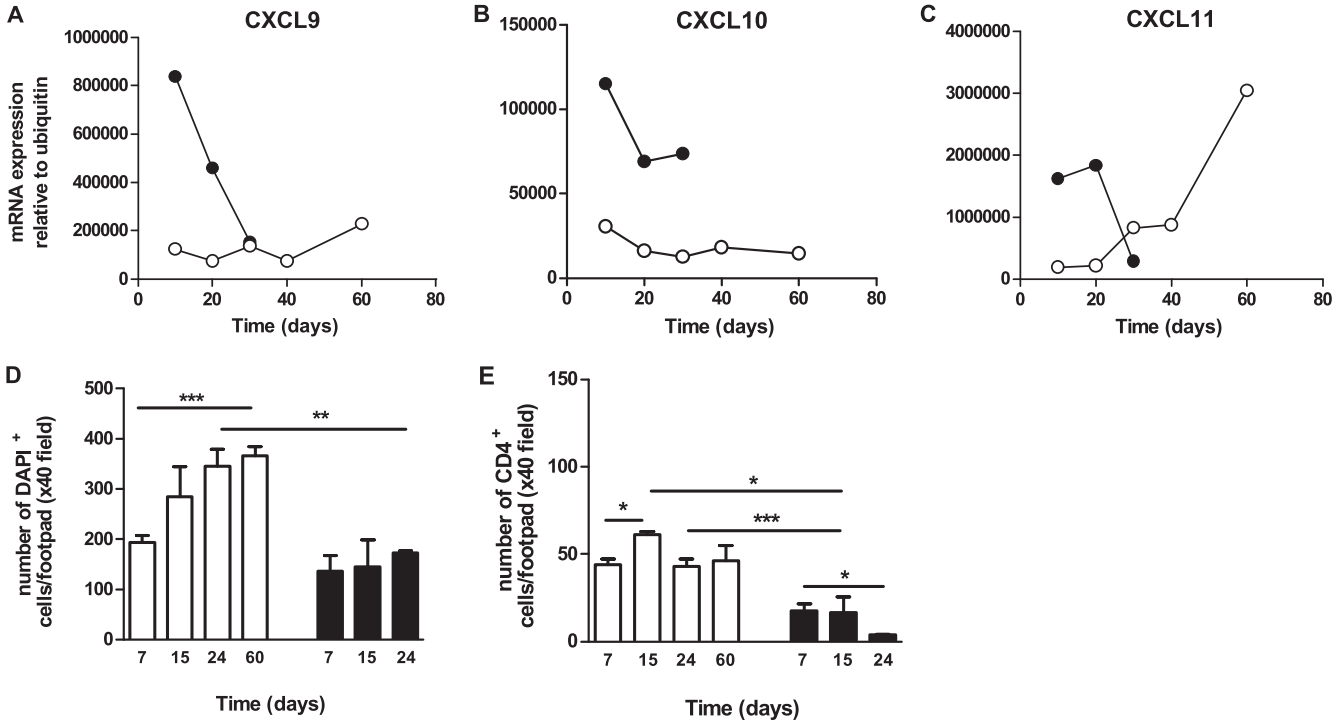

FIG. 5. Early recruitment followed by depletion of $CD4^+$ cells in the footpad during infection with highly virulent *M. ulcerans*. Mice were subcutaneously infected in the footpad with 3×10^5 AFB of *M. ulcerans* 5114 (○ or white bars) or 98-912 (● or black bars). (A to C) At specific time points, RNA was extracted from a single cell suspension of the footpad. The presence of specific mRNAs for CXCL9, CXCL10, and CXCL11 were determined by real-time PCR. The data points represent the means ± the SEM ($n = 5$) for each time point. The results are from one experiment representative of two separate experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (D and E) Tissue sections of the footpad were stained with DAPI (D) and anti-CD4 (E). The average number of $DAPI^+$ or $CD4^+$ cells per footpad was determined by using a 40× objective lens. The data points represent the means ± the SEM ($n = 4$). The results are from one experiment representative of two independent experiments.

counts of *L. monocytogenes* in both the spleen and liver (Fig. 7). These results show that the late local and regional immunosuppression occurring during active infection with a virulent mycolactone-producing *M. ulcerans* strain is not accompanied by a systemic suppression of CMI.

DISCUSSION

The initiation of a protective immune response against intracellular pathogens requires the activation and migration of antigen-presenting cells to the DLN to achieve T-cell priming (45, 73). Mycolactone has been reported to inhibit macrophage responses (4, 12, 40, 44, 54, 65, 67), the maturation and/or migration of dendritic cells, and consequently T-cell priming (11). Moreover, it has been recently reported that mycolactone injected subcutaneously or produced during experimental *M. ulcerans* infection was detected in lymphoid organs and in blood mononuclear cells. In addition, this diffusion of mycolactone was associated with decreased production of IL-2 by circulating lymphocytes (25). As a consequence, it has been suggested that *M. ulcerans* infections induce anergy and systemic immunosuppression (11, 19–21, 25, 75).

By experimentally infecting immunodeficient mice, we show here that T cells are involved in the control of infection by a low-virulence *M. ulcerans* strain but eventually are not effective against a highly virulent mycolactone-producing strain. Nevertheless, infection with *M. ulcerans* 98-912, and the consequent production of mycolactone, did not inhibit the development of an OVA-specific response in the DLN, showing that antigen presentation and T-cell priming is not impaired. In addition, we showed that at the initial phase of infection, expression of IFN-γ and development of mycobacterium-specific IFN-γ-producing T cells occurred in the DLN. In fact, infection with highly virulent, mycolactone-producing *M. ulcerans* triggered a stronger T-cell response in comparison to a low virulent, mycolactone-negative strain, which may be related to a stronger inflammatory stimulus associated with the higher bacterial load. The occurrence of an initial adaptive CMI immune response in the DLN is in accordance with the resistance to *M. ulcerans* infection being associated with IFN-γ production in humans (20, 21, 42, 48, 70, 72) and in experimental infections (61, 67) and with the concept that *M. ulcerans* is an intracellular pathogen (53, 66).

Moreover, the $CD4^+$ T cells activated in the DLN of mice infected with either *M. ulcerans* strains migrate to the peripheral infectious focus, mobilized by Th1-recruiting chemokines. However, while $CD4^+$ T cells persist in the focus of infection with the low-virulence strain, they are progressively depleted during infection with strain 98-912, correlating with extensive leukocyte apoptosis and tissue necrosis (35). This depletion of $CD4^+$ T cells in the footpad was already observed at day 15 postinfection, when their numbers in the DLN were still in-

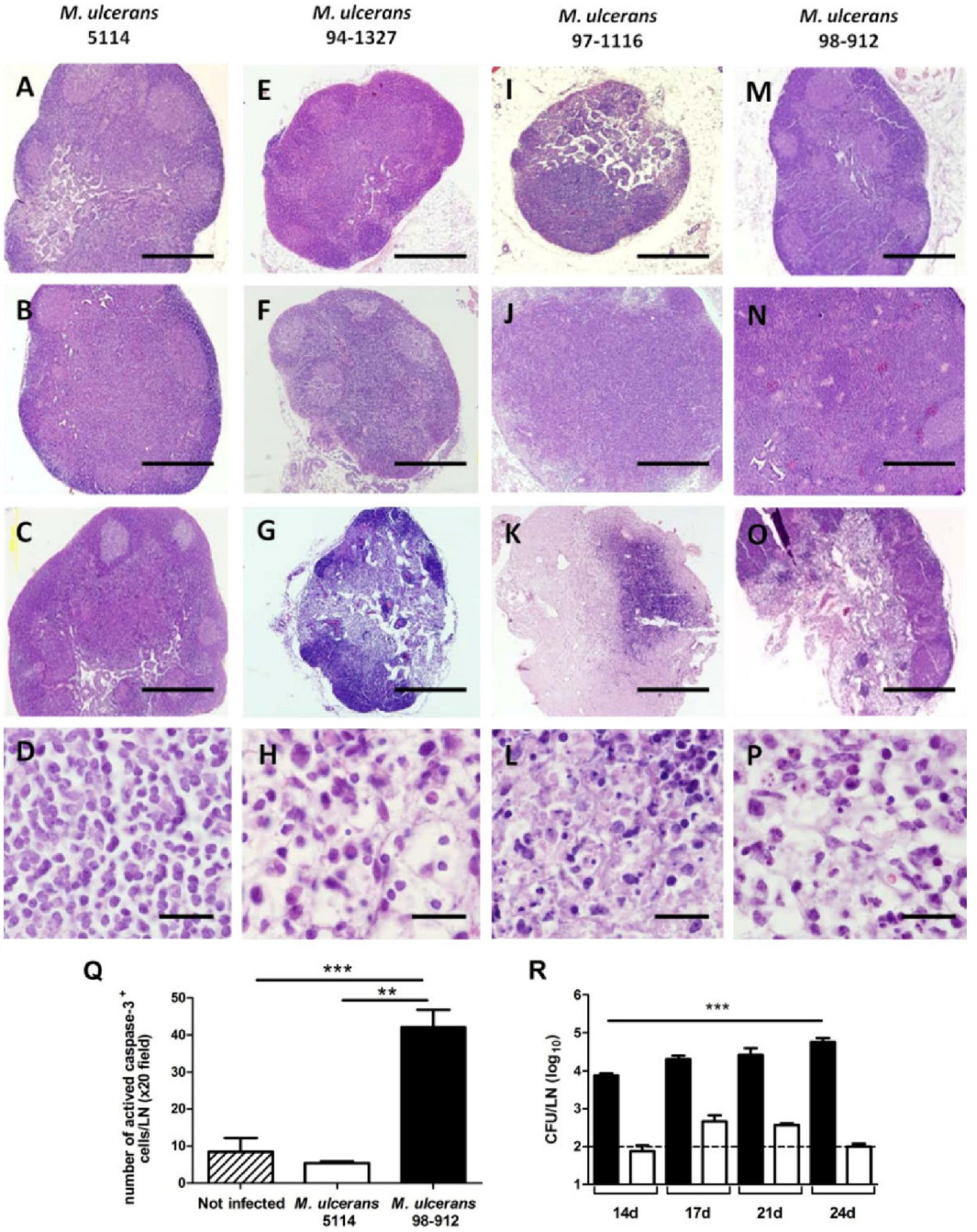

FIG. 6. Late apoptotic destruction of the DLN in mice infected with highly virulent *M. ulcerans* is associated with a higher bacterial burden. The DLN of mice subcutaneously infected in the footpad with 3×10^5 AFB of *M. ulcerans* 5114 (A to D), 94-1327 (E to H), 97-1116 (I to L), or 98-912 (M to P) were harvested at day 10 (A, E, I, and M), 15 (B and N), 20 (F, J, O, and P), 50 (K and L), and 60 (C, D, G, and H). Tissue sections were stained with H&E. The images are representative of five lymph nodes per group analyzed in four independent experiments. For panels A to C, E to G, I to K, and M to O, the scale bars represent 500 μm. For panels D, H, L, and P, the scale bars represent 20 μm. (Q) The DLN of noninfected mice (dashed bar) or mice infected with *M. ulcerans* 5114 (white bar) or 98-912 (black bar) were harvested at day 15. Apoptotic cells were labeled by immunofluorescence for activated caspase 3. The average number of activated caspase 3^+ cells/LN was determined by using a 20× objective lens. The data points represent the means ± the SEM ($n = 4$). The results are from one of two independent experiments. (R) The bacterial burden of DLN of mice infected with *M. ulcerans* 5114 (white bars) or 98-912 (black bars) was determined over time by CFU counts. Dashed line represents the detection limit. The data points represent the means ± the SEM ($n = 5$). The results are from one of two separate experiments. Statistical significance was calculated using the Student *t* test (**, $P < 0.01$; ***, $P < 0.001$).

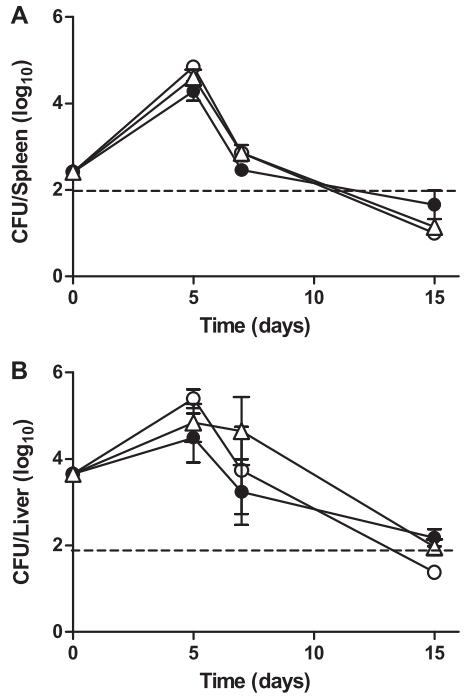

FIG. 7. Subcutaneous footpad infection with a highly virulent strain of *M. ulcerans* does not increase the susceptibility to a systemic coinfection with *L. monocytogenes*. Mice were either left uninfected (△) or subcutaneously infected in the footpad with 3×10^5 AFB of *M. ulcerans* 5114 (○) or 98-912 (●). During the fourth week of infection, when the infected footpad showed evidence of advanced swelling, mice were intravenously infected with 5×10^3 CFU of *L. monocytogenes*. The spleens and livers were harvested, and the bacterial burden (CFU counts) was determined over time. The dashed line represents the detection limit. The data points represent the means ± the SEM (n = 5). The results are from one experiment representative of two separate experiments.

creasing, which is likely due to an earlier accumulation of mycolactone at the initial site of infection.

Collectively, our results show that the early recruitment of T cells to the primary site of infection is not compromised by infection with high virulent *M. ulcerans*; however, in time this potentially protective response is abrogated by local and regional suppression of CMI. The lack of a sustained adaptive immune response to virulent *M. ulcerans* may have the contribution of other factors, namely, (i) mycolactone-mediated inhibition of antimycobacterial mechanisms in IFN-γ-activated macrophages (67), (ii) mycolactone-induced dysregulation of intracellular signaling pathways coupled to TCR activation (6), and (iii) vascular pathology with occlusion of vessels as occurs in progressive BU lesions (23, 29, 53, 71).

By the end of the experimental infection with the mycolactone-positive strain, we observed a decrease in the total number of cells in the DLN. This local/regional cytotoxic effect of mycolactone does not seem to be specific for T cells, since B cells were equally affected. This tissue destruction of lymph nodes was associated with bacterial colonization, which is consistent with the spreading of *M. ulcerans* from the site of infection to the lymph nodes via afferent lymphatic drainage (3, 12, 74). Lymphadenopathy in BU patients has been seldom appreciated; however, there is a description of lymph node destruction and bacterial colonization (74), confirming our experimental results. The continuous lymphatic dissemination of *M. ulcerans*, either freely or shuttled in phagocytes, could explain the high recurrence rates after surgical intervention alone (8, 34, 46), as well as cases of disseminated BU (1, 9, 24, 43, 57). Our results highlight the importance of investigating lymph node involvement in BU patients and considering the impact of possible bacterial colonization during treatment procedures, further reinforcing the importance of antibiotic therapy.

Previous studies have suggested that *M. ulcerans* infection leads to the occurrence of systemic anergy/immunosuppression. This interpretation was mainly based on reports of lack of response of antigen-specific peripheral blood mononuclear cells from active BU patients upon specific stimuli (19–21, 42, 75). Mycolactone was further implicated in this status of peripheral anergy/immunosuppression, when the toxin was detected in lymphoid organs and in blood mononuclear cells during an experimental subcutaneous infection with *M. ulcerans* (25). However, our observation of DLN destruction, with consequent depletion of T cells, could explain the lack of specific T-cell responses reported in the periphery. Moreover, we show that progressive, advanced infection with *M. ulcerans* did not render mice more susceptible to a systemic coinfection with *L. monocytogenes*, an infection for which the host requires T-cell-mediated immunity for protection (5, 33, 37). This further supports our interpretation that the local and regional destruction of immune cells that occurs during *M. ulcerans* infection is not associated with systemic immunosuppression. This is in line with BU patients not having an increased susceptibility to opportunistic infections and with cutaneous BU being associated with minimum systemic effects (70).

The lack of knowledge about the amount of cytotoxic factors released from *M. ulcerans* during the early or late stages of active infection has already been discussed (53), but our present data suggest that, although mycolactone might reach peripheral organs, its biological activity is probably limited to sites where its concentration is enough to produce cytopathology (53).

In addition to mycolactone, other macrolides have been described as immunosuppressors. FK605 is an immunosuppressive macrolide mainly used to lower the risk of allogeneic transplant rejection by inhibiting T-cell signal transduction and interleukin-2 transcription (30) and by affecting dendritic cell differentiation and function (2, 22). Although FK506 is structurally similar to mycolactone, it has been suggested that the mechanisms underlying mycolactone's suppressive activity are different and that this specificity of action may define a novel class of immunosuppressive agents (11). However, our observations do not support a systemic immunosuppressive effect of mycolactone, at least with the amounts produced during a footpad *M. ulcerans* infection.

The findings reported here contribute to foster our understanding of BU pathogenesis and have important implications for the design of new prophylactic/therapeutic strategies. Indeed, our data suggest that early antibiotic treatment is essential to prevent the compromise of the early development of IFN-γ-mediated CMI. Furthermore, our results prompt the development of improved vaccines that would boost this type of adaptive antibacterial immunity, leading to an early recall

immune response before the build-up of mycolactone compromises the T-cell differentiation and persistence.

ACKNOWLEDGMENTS

This study was supported by grants from the Health Services of Fundação Calouste Gulbenkian, and by the Portuguese Science and Technology Foundation (FCT). A. Fraga was supported by the FCT fellowship SFRH/BD/15911/2005.

REFERENCES

1. **Abalos, F. M., J. Aguiar, Sr., A. Guedenon, F. Portaels, and W. M. Meyers.** 2000. *Mycobacterium ulcerans* infection (Buruli ulcer): a case report of the disseminated nonulcerative form. Ann. Diagn. Pathol. **4:**386–390.
2. **Abe, M., and A. W. Thomson.** 2003. Influence of immunosuppressive drugs on dendritic cells. Transpl. Immunol. **11:**357–365.
3. **Addo, P., E. Owusu, B. Adu-Addai, M. Quartey, M. Abbas, A. Dodoo, and D. Ofori-Adjei.** 2005. Findings from a buruli ulcer mouse model study. Ghana Med. J. **39:**86–93.
4. **Adusumilli, S., A. Mve-Obiang, T. Sparer, W. Meyers, J. Hayman, and P. L. Small.** 2005. *Mycobacterium ulcerans* toxic macrolide, mycolactone, modulates the host immune response and cellular location of *M. ulcerans* in vitro and in vivo. Cell Microbiol. **7:**1295–1304.
5. **Bancroft, G. J., R. D. Schreiber, and E. R. Unanue.** 1991. Natural immunity: a T-cell-independent pathway of macrophage activation, defined in the scid mouse. Immunol. Rev. **124:**5–24.
6. **Boulkroun, S., L. Guenin-Mace, M. I. Thoulouze, M. Monot, A. Merckx, G. Langsley, G. Bismuth, V. Di Bartolo, and C. Demangel.** 2010. Mycolactone suppresses T-cell responsiveness by altering both early signaling and post-translational events. J. Immunol. **184:**1436–1444.
7. **Castro, A. G., M. Neighbors, S. D. Hurst, F. Zonin, R. A. Silva, E. Murphy, Y. J. Liu, and A. O'Garra.** 2000. Anti-interleukin 10 receptor monoclonal antibody is an adjuvant for T helper cell type 1 responses to soluble antigen only in the presence of lipopolysaccharide. J. Exp. Med. **192:**1529–1534.
8. **Chauty, A., M. F. Ardant, A. Adeye, H. Euverte, A. Guedenon, C. Johnson, J. Aubry, E. Nuermberger, and J. Grosset.** 2007. Promising clinical efficacy of streptomycin-rifampin combination for treatment of buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob. Agents Chemother. **51:**4029–4035.
9. **Coloma, J. N., G. Navarrete-Franco, P. Iribe, and L. D. Lopez-Cepeda.** 2005. Ulcerative cutaneous mycobacteriosis due to *Mycobacterium ulcerans*: report of two Mexican cases. Int. J. Lepr. Other Mycobact. Dis. **73:**5–12.
10. **Connor, D. H., and H. F. Lunn.** 1965. *Mycobacterium ulcerans* infection (with comments on pathogenesis). Int. J. Lepr. **33**(Suppl.)**:**698–709.
11. **Coutanceau, E., J. Decalf, A. Martino, A. Babon, N. Winter, S. T. Cole, M. L. Albert, and C. Demangel.** 2007. Selective suppression of dendritic cell functions by *Mycobacterium ulcerans* toxin mycolactone. J. Exp. Med. **204:**1395–1403.
12. **Coutanceau, E., L. Marsollier, R. Brosch, E. Perret, P. Goossens, M. Tanguy, S. T. Cole, P. L. Small, and C. Demangel.** 2005. Modulation of the host immune response by a transient intracellular stage of *Mycobacterium ulcerans*: the contribution of endogenous mycolactone toxin. Cell Microbiol. **7:**1187–1196.
13. **Cruz, A., S. A. Khader, E. Torrado, A. Fraga, J. E. Pearl, J. Pedrosa, A. M. Cooper, and A. G. Castro.** 2006. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. J. Immunol. **177:**1416–1420.
14. **Diaz, D., H. Dobeli, D. Yeboah-Manu, E. Mensah-Quainoo, A. Friedlein, N. Soder, S. Rondini, T. Bodmer, and G. Pluschke.** 2006. Use of the immunodominant 18-kiloDalton small heat shock protein as a serological marker for exposure to *Mycobacterium ulcerans*. Clin. Vaccine Immunol. **13:**1314–1321.
15. **Dobos, K. M., E. A. Spotts, B. J. Marston, C. R. Horsburgh, Jr., and C. H. King.** 2000. Serologic response to culture filtrate antigens of *Mycobacterium ulcerans* during Buruli ulcer disease. Emerg. Infect. Dis. **6:**158–164.
16. **Fenner, F.** 1957. Homologous and heterologous immunity in infections of mice with *Mycobacterium ulcerans* and *Mycobacterium balnei*. Am. Rev. Tuberc. **76:**76–89.
17. **George, K. M., D. Chatterjee, G. Gunawardana, D. Welty, J. Hayman, R. Lee, and P. L. Small.** 1999. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science **283:**854–857.
18. **George, K. M., L. Pascopella, D. M. Welty, and P. L. Small.** 2000. A *Mycobacterium ulcerans* toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. Infect. Immun. **68:**877–883.
19. **Gooding, T. M., P. D. Johnson, D. E. Campbell, J. A. Hayman, E. L. Hartland, A. S. Kemp, and R. M. Robins-Browne.** 2001. Immune response to infection with *Mycobacterium ulcerans*. Infect. Immun. **69:**1704–1707.
20. **Gooding, T. M., P. D. Johnson, M. Smith, A. S. Kemp, and R. M. Robins-Browne.** 2002. Cytokine profiles of patients infected with *Mycobacterium ulcerans* and unaffected household contacts. Infect. Immun. **70:**5562–5567.
21. **Gooding, T. M., A. S. Kemp, R. M. Robins-Browne, M. Smith, and P. D. Johnson.** 2003. Acquired T-helper 1 lymphocyte anergy following infection with *Mycobacterium ulcerans*. Clin. Infect. Dis. **36:**1076–1077.
22. **Hackstein, H., T. Taner, A. F. Zahorchak, A. E. Morelli, A. J. Logar, A. Gessner, and A. W. Thomson.** 2003. Rapamycin inhibits IL-4-induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. Blood **101:**4457–4463.
23. **Hayman, J.** 1993. Out of Africa: observations on the histopathology of *Mycobacterium ulcerans* infection. J. Clin. Pathol. **46:**5–9.
24. **Hofer, M., B. Hirschel, P. Kirschner, M. Beghetti, A. Kaelin, C. A. Siegrist, S. Suter, A. Teske, and E. C. Bottger.** 1993. Brief report: disseminated osteomyelitis from *Mycobacterium ulcerans* after a snakebite. N. Engl. J. Med. **328:**1007–1009.
25. **Hong, H., E. Coutanceau, M. Leclerc, L. Caleechurn, P. F. Leadlay, and C. Demangel.** 2008. Mycolactone diffuses from *Mycobacterium ulcerans*-infected tissues and targets mononuclear cells in peripheral blood and lymphoid organs. PLoS Negl. Trop. Dis. **2:**e325.
26. **Hong, H., J. B. Spencer, J. L. Porter, P. F. Leadlay, and T. Stinear.** 2005. A novel mycolactone from a clinical isolate of *Mycobacterium ulcerans* provides evidence for additional toxin heterogeneity as a result of specific changes in the modular polyketide synthase. Chembiochem **6:**643–648.
27. **Johnson, R. C., F. Nackers, J. R. Glynn, E. de Biurrun Bakedano, C. Zinsou, J. Aguiar, R. Tonglet, and F. Portaels.** 2008. Association of HIV infection and *Mycobacterium ulcerans* disease in Benin. AIDS **22:**901–903.
28. **Kiszewski, A. E., E. Becerril, L. D. Aguilar, I. T. Kader, W. Myers, F. Portaels, and R. Hernandez Pando.** 2006. The local immune response in ulcerative lesions of Buruli disease. Clin. Exp. Immunol. **143:**445–451.
29. **Krieg, R. E., W. T. Hockmeyer, and D. H. Connor.** 1974. Toxin of *Mycobacterium ulcerans*: production and effects in guinea pig skin. Arch. Dermatol. **110:**783–788.
30. **Liu, J., J. D. Farmer, Jr., W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber.** 1991. Calcineurin is a common target of cyclophilin-cyclosporine A and FKBP-FK506 complexes. Cell **66:**807–815.
31. **Marston, B. J., M. O. Diallo, C. R. Horsburgh, Jr., I. Diomande, M. Z. Saki, J. M. Kanga, G. Patrice, H. B. Lipman, S. M. Ostroff, and R. C. Good.** 1995. Emergence of Buruli ulcer disease in the Daloa region of Cote d'Ivoire. Am. J. Trop. Med. Hyg. **52:**219–224.
32. **Mve-Obiang, A., R. E. Lee, F. Portaels, and P. L. Small.** 2003. Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: implications for virulence. Infect. Immun. **71:**774–783.
33. **Nickol, A. D., and P. F. Bonventre.** 1977. Anomalous high native resistance to athymic mice to bacterial pathogens. Infect. Immun. **18:**636–645.
34. **Nienhuis, W. A., Y. Stienstra, W. A. Thompson, P. C. Awuah, K. M. Abass, W. Tuah, N. Y. Awua-Boateng, E. O. Ampadu, V. Siegmund, J. P. Schouten, O. Adjei, G. Bretzel, and T. S. van der Werf.** 2010. Antimicrobial treatment for early, limited *Mycobacterium ulcerans* infection: a randomised controlled trial. Lancet **375:**664–672.
35. **Oliveira, M. S., A. G. Fraga, E. Torrado, A. G. Castro, J. P. Pereira, A. L. Filho, F. Milanezi, F. C. Schmitt, W. M. Meyers, F. Portaels, M. T. Silva, and J. Pedrosa.** 2005. Infection with *Mycobacterium ulcerans* induces persistent inflammatory responses in mice. Infect. Immun. **73:**6299–6310.
36. **Olson, T. S., and K. Ley.** 2002. Chemokines and chemokine receptors in leukocyte trafficking. Am. J. Physiol. Regul. Integr. Comp. Physiol. **283:**R7–R28.
37. **Pamer, E. G.** 2004. Immune responses to *Listeria monocytogenes*. Nat. Rev. Immunol. **4:**812–823.
38. **Peduzzi, E., C. Groeper, D. Schutte, P. Zajac, S. Rondini, E. Mensah-Quainoo, G. C. Spagnoli, G. Pluschke, and C. A. Daubenberger.** 2007. Local activation of the innate immune system in Buruli ulcer lesions. J. Invest. Dermatol. **127:**638–645.
39. **Pelleitier, M., and S. Montplaisir.** 1975. The nude mouse: a model of deficient T-cell function. Methods Achiev. Exp. Pathol. **7:**149–166.
40. **Pimsler, M., T. A. Sponsler, and W. M. Meyers.** 1988. Immunosuppressive properties of the soluble toxin from *Mycobacterium ulcerans*. J. Infect. Dis. **157:**577–580.
41. **Portaels, F., M. T. Silva, and W. M. Meyers.** 2009. Buruli ulcer. Clin. Dermatol. **27:**291–305.
42. **Prevot, G., E. Bourreau, H. Pascalis, R. Pradinaud, A. Tanghe, K. Huygen, and P. Launois.** 2004. Differential production of systemic and intralesional gamma interferon and interleukin-10 in nodular and ulcerative forms of Buruli disease. Infect. Immun. **72:**958–965.
43. **Pszolla, N., M. R. Sarkar, W. Strecker, P. Kern, L. Kinzl, W. M. Meyers, and F. Portaels.** 2003. Buruli ulcer: a systemic disease. Clin. Infect. Dis. **37:**e78–e82.
44. **Rastogi, N., M. C. Blom-Potar, and H. L. David.** 1989. Comparative intracellular growth of difficult-to-grow and other mycobacteria in a macrophage cell line. Acta Leprol. **7**(Suppl. 1)**:**156–159.
45. **Reiley, W. W., M. D. Calayag, S. T. Wittmer, J. L. Huntington, J. E. Pearl, J. J. Fountain, C. A. Martino, A. D. Roberts, A. M. Cooper, G. M. Winslow, and D. L. Woodland.** 2008. ESAT-6-specific CD4 T-cell responses to aerosol *Mycobacterium tuberculosis* infection are initiated in the mediastinal lymph nodes. Proc. Natl. Acad. Sci. U. S. A. **105:**10961–10966.
46. **Sarfo, F. S., R. Phillips, K. Asiedu, E. Ampadu, N. Bobi, E. Adentwe, A. Lartey, I. Tetteh, and M. Wansbrough-Jones.** 2010. Clinical efficacy of com-

bination of rifampin and streptomycin for treatment of *Mycobacterium ulcerans* disease. Antimicrob. Agents Chemother. **54:**3678–3685.
47. **Sasaki, T., M. Mieno, H. Udono, K. Yamaguchi, T. Usui, K. Hara, H. Shiku, and E. Nakayama.** 1990. Roles of $CD4^+$ and $CD8^+$ cells, and the effect of administration of recombinant murine interferon gamma in listerial infection. J. Exp. Med. **171:**1141–1154.
48. **Schipper, H. S., B. Rutgers, M. G. Huitema, S. N. Etuaful, B. D. Westenbrink, P. C. Limburg, W. Timens, and T. S. van der Werf.** 2007. Systemic and local interferon-gamma production following *Mycobacterium ulcerans* infection. Clin. Exp. Immunol. **150:**451–459.
49. **Schutte, D., A. Um-Boock, E. Mensah-Quainoo, P. Itin, P. Schmid, and G. Pluschke.** 2007. Development of highly organized lymphoid structures in Buruli ulcer lesions after treatment with rifampicin and streptomycin. PLoS Negl. Trop. Dis. **1:**e2.
50. **Shepard, C. C., and D. H. McRae.** 1968. A method for counting acid-fast bacteria. Int. J. Lepr. Other Mycobact. Dis. **36:**78–82.
51. **Shinkai, Y., S. Koyasu, K. Nakayama, K. M. Murphy, D. Y. Loh, E. L. Reinherz, and F. W. Alt.** 1993. Restoration of T-cell development in RAG-2-deficient mice by functional TCR transgenes. Science **259:**822–825.
52. **Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, et al.** 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J. rearrangement. Cell **68:**855–867.
53. **Silva, M. T., F. Portaels, and J. Pedrosa.** 2009. Pathogenetic mechanisms of the intracellular parasite *Mycobacterium ulcerans* leading to Buruli ulcer. Lancet Infect. Dis. **9:**699–710.
54. **Simmonds, R. E., F. V. Lali, T. Smallie, P. L. Small, and B. M. Foxwell.** 2009. Mycolactone inhibits monocyte cytokine production by a posttranscriptional mechanism. J. Immunol. **182:**2194–2202.
55. **Smith, P. G., W. D. Revill, E. Lukwago, and Y. P. Rykushin.** 1976. The protective effect of BCG against *Mycobacterium ulcerans* disease: a controlled trial in an endemic area of Uganda. Trans. R. Soc. Trop. Med. Hyg. **70:**449–457.
56. **Snyder, D. S., and P. L. Small.** 2003. Uptake and cellular actions of mycolactone, a virulence determinant for *Mycobacterium ulcerans*. Microb. Pathog. **34:**91–101.
57. **Sopoh, G. E., A. D. Dossou, L. V. Brun, Y. T. Barogui, J. G. Houezo, D. Affolabi, S. Y. Anagonou, R. C. Johnson, L. Kestens, and F. Portaels.** 2010. Severe multifocal form of buruli ulcer after streptomycin and rifampin treatment: comments on possible dissemination mechanisms. Am. J. Trop. Med. Hyg. **83:**307–313.
58. **Stienstra, Y., W. T. van der Graaf, G. J. te Meerman, T. H. The, L. F. de Leij, and T. S. van der Werf.** 2001. Susceptibility to development of *Mycobacterium ulcerans* disease: review of possible risk factors. Trop. Med. Int. Health **6:**554–562.
59. **Stinear, T. P., H. Hong, W. Frigui, M. J. Pryor, R. Brosch, T. Garnier, P. F. Leadlay, and S. T. Cole.** 2005. Common evolutionary origin for the unstable virulence plasmid pMUM found in geographically diverse strains of *Mycobacterium ulcerans*. J. Bacteriol. **187:**1668–1676.
60. **Tanghe, A., P. Y. Adnet, T. Gartner, and K. Huygen.** 2007. A booster vaccination with *Mycobacterium bovis* BCG does not increase the protective effect of the vaccine against experimental *Mycobacterium ulcerans* infection in mice. Infect. Immun. **75:**2642–2644.
61. **Tanghe, A., J. Content, J. P. Van Vooren, F. Portaels, and K. Huygen.** 2001. Protective efficacy of a DNA vaccine encoding antigen 85A from *Mycobacterium bovis* BCG against Buruli ulcer. Infect. Immun. **69:**5403–5411.
62. **Tanghe, A., J. P. Dangy, G. Pluschke, and K. Huygen.** 2008. Improved protective efficacy of a species-specific DNA vaccine encoding mycolyl-transferase Ag85A from *Mycobacterium ulcerans* by homologous protein boosting. PLoS Negl. Trop. Dis. **2:**e199.
63. **Thangaraj, H. S., M. R. Evans, and M. H. Wansbrough-Jones.** 1999. *Mycobacterium ulcerans* disease; Buruli ulcer. Trans. R. Soc. Trop. Med. Hyg. **93:**337–340.
64. **Toll, A., F. Gallardo, M. Ferran, M. Gilaberte, M. Iglesias, J. L. Gimeno, S. Rondini, and R. M. Pujol.** 2005. Aggressive multifocal Buruli ulcer with associated osteomyelitis in an HIV-positive patient. Clin. Exp. Dermatol. **30:**649–651.
65. **Torrado, E., S. Adusumilli, A. G. Fraga, P. L. Small, A. G. Castro, and J. Pedrosa.** 2007. Mycolactone-mediated inhibition of tumor necrosis factor production by macrophages infected with *Mycobacterium ulcerans* has implications for the control of infection. Infect. Immun. **75:**3979–3988.
66. **Torrado, E., A. G. Fraga, A. G. Castro, P. Stragier, W. M. Meyers, F. Portaels, M. T. Silva, and J. Pedrosa.** 2007. Evidence for an intramacrophage growth phase of *Mycobacterium ulcerans*. Infect. Immun. **75:**977–987.
67. **Torrado, E., A. G. Fraga, E. Logarinho, T. G. Martins, J. A. Carmona, J. B. Gama, M. A. Carvalho, F. Proenca, A. G. Castro, and J. Pedrosa.** 2010. IFN-γ-dependent activation of macrophages during experimental infections by *Mycobacterium ulcerans* is impaired by the toxin mycolactone. J. Immunol. **184:**947–955.
68. **Uganda Buruli Group.** 1969. BCG vaccination against *Mycobacterium ulcerans* infection (Buruli ulcer): first results of a trial in Uganda. Lancet **i:**111–115.
69. **van der Werf, T. S., Y. Stienstra, R. C. Johnson, R. Phillips, O. Adjei, B. Fleischer, M. H. Wansbrough-Jones, P. D. Johnson, F. Portaels, W. T. van der Graaf, and K. Asiedu.** 2005. *Mycobacterium ulcerans* disease. Bull. World Health Organ. **83:**785–791.
70. **van der Werf, T. S., W. T. van der Graaf, J. W. Tappero, and K. Asiedu.** 1999. *Mycobacterium ulcerans* infection. Lancet **354:**1013–1018.
71. **Walsh, D. S., F. Portaels, and W. M. Meyers.** 2008. Buruli ulcer (*Mycobacterium ulcerans* infection). Trans. R. Soc. Trop. Med. Hyg. **102:**969–978.
72. **Westenbrink, B. D., Y. Stienstra, M. G. Huitema, W. A. Thompson, E. O. Klutse, E. O. Ampadu, H. M. Boezen, P. C. Limburg, and T. S. van der Werf.** 2005. Cytokine responses to stimulation of whole blood from patients with Buruli ulcer disease in Ghana. Clin. Diagn. Lab. Immunol. **12:**125–129.
73. **Wolf, A. J., L. Desvignes, B. Linas, N. Banaiee, T. Tamura, K. Takatsu, and J. D. Ernst.** 2008. Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. J. Exp. Med. **205:**105–115.
74. **World Health Organization.** 2000. Buruli ulcer, *Mycobacterium ulcerans* infection. WHO/CDS/CPEGBUI/2000.1. World Health Organization, Geneva, Switzerland.
75. **Yeboah-Manu, D., E. Peduzzi, E. Mensah-Quainoo, A. Asante-Poku, D. Ofori-Adjei, G. Pluschke, and C. A. Daubenberger.** 2006. Systemic suppression of interferon-gamma responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen *Mycobacterium ulcerans*. J. Leukoc. Biol. **79:**1150–1156.

Editor: J. L. Flynn