MAJOR ARTICLE

Immunosuppressive Signature of Cutaneous *Mycobacterium ulcerans* Infection in the Peripheral Blood of Patients with Buruli Ulcer Disease

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Buruli ulcer disease (BUD) is an emerging human disease caused by infection with *Mycobacterium ulcerans,* which leads to the development of necrotic skin lesions. The pathogenesis of the ulcer is closely associated with the production of mycolactone, a diffusible cytotoxin with immunomodulatory properties. To identify immunological correlates of BUD, we performed a broad screen of inflammatory mediators in serum samples and stimulated whole-blood supernatants of patients. We found that patients with active ulcers displayed a distinctive profile of immune suppression, marked by the down-modulation of selected chemokines and an impaired capacity to produce Th1, Th2, and Th17 cytokines on stimulation with mitogenic agents. These immunological defects were induced early in the disease and resolved after anti-BUD therapy, establishing their association with the presence of *M. ulcerans*. Interestingly, some of the defects in cytokine and chemokine response could be mimicked in vitro by incubation of $CD4^+$ peripheral blood lymphocytes with mycolactone. Our findings support the hypothesis that mycolactone contributes to bacterial persistence in human hosts by limiting the generation of adaptive cellular responses. Moreover, we identified immunological markers of BUD, which may be helpful for confirmatory diagnosis of the disease and, especially, for monitoring the response to antibiotic treatment.

Buruli ulcer disease (BUD) is a disease of subcutaneous tissue caused by infection with *Mycobacterium ulcerans,* from the family of bacteria causing tuberculosis and leprosy [1]. BUD starts as a painless nodule, papule, or plaque, which eventually breaks down centrally to form a characteristic ulcer with undermined edges. The extensive tissue destruction associated with BUD lesions often leads to gross deformities and permanent disability. Since the late 1980s, the prevalence of the disease has been increasing dramatically in West and Central Africa, prompting the World Health Organization (WHO) to initiate an awareness and control campaign in 1998 [2]. BUD is usually diagnosed on the basis of clinical symptoms, because identification of *M. ulcerans* by means of cultures or polymerase chain reaction (PCR) requires dedicated facilities and specialized equipment. Recently, major advances have been made in the treatment of the disease with antibiotics [3, 4]. However, although antimycobacterial drugs are relatively effective during the preulcerative stage of the disease, antibiotic treatment requires optimization, and surgical excision with skin grafting remains the only alternative for advanced lesions. To control the emergence of BUD, alternative diagnostic approaches that allow the early identification of patients and better biomarkers of the infection are clearly needed.

A distinctive feature of *M. ulcerans* is the production

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Table 1. Characteristics of Patients with Buruli Ulcer Disease (BUD), Control Participants, and Patients with Tuberculosis (TB)

Characteristic	Patients with BUD			Healthy control ($n = 21$)[c]	Patients with TB ($n = 8$)[c]
	Preulcerative ($n = 13$)[a]	Ulcerative ($n = 15$)	Kinetic study ($n = 9$)[b]		
Age, median (range), years	14 (5–45)	16 (8–40)	10 (7–25)	27 (12–56)	38 (24–61)
Sex, no. male/no. female	7/5	5/10	3/6	8/13	4/4

[a] Patients in the preulcerative group had disease characterized by nodule, edema, or plaque.
[b] The kinetic study included patients with BUD who were receiving antibiotic treatment.
[c] Control participants and patients with tuberculosis were from the same zone of endemicity as were patients with BUD.

of a macrocyclic polyketide called mycolactone [5]. Mycolactone plays a critical role in bacterial pathogenicity, as shown by the necrotic lesions induced by intradermal injection of the purified molecule [6]. *M. ulcerans* bacilli remain essentially localized in peripheral subcutaneous tissues, but there is evidence from animal studies that mycolactone diffuses from infectious foci into the blood, where it accumulates in mononuclear cell subsets [7]. Although mycolactone is cytotoxic and proapoptotic [8, 9], recent data suggest that it plays a major role in allowing *M. ulcerans* to evade host innate immunity by suppressing inflammatory processes. In support of this hypothesis, noncytotoxic doses of mycolactone were found to block the capacity of dendritic cells to prime cellular immune responses and produce chemotactic signals of inflammation in vitro [10]. Furthermore, macrophages and monocytes treated by mycolactone before stimulation failed to produce several inflammatory cytokines, without loss of cell viability [11–14]. Interestingly, *M. ulcerans* lipid extracts suppress the activation-induced production of interleukin (IL) 2 by human T cell lines. Moreover, systemic interferon (IFN) γ responses are modulated in patients during BUD progression [15–21], suggesting that mycolactone may also interfere with the functional biology of T cells in vivo.

In the present study, we searched for immunological markers of *M. ulcerans* infection in patient serum and evaluated whether these could help improve current approaches to BUD diagnosis and treatment. We found that, in contrast to healthy participants and patients with pulmonary tuberculosis, patients with BUD displayed a characteristic suppression of circulating chemokines. In addition, the capacity of their lymphocytes to produce inflammatory cytokines in ex vivo stimulation assays was largely impaired. Some of the immunological defects of patients with BUD could be reproduced in vitro by treating $CD4^+$ T cells with mycolactone, which would identify a novel cell subset targeted by this immunosuppressive agent and support the hypothesis that mycolactone limits the generation of host adaptive cellular responses by suppressing $CD4^+$ T cell effector functions. Therefore, in addition to providing novel information on the pathogenesis of BUD, our study identified biomarkers of *M. ulcerans* infection that correlate with disease progression.

MATERIALS AND METHODS

Mycolactone preparation. *M. ulcerans* 1615 (American Type Culture Collection 35840), which produces a mixture of mycolactones A/B and C, was obtained from the Trudeau Institute collection. Bacteria were cultivated in Dubos medium complemented with 10% oleic acid–albumin-dextrose-catalase (Becton Dickinson) in spinner flasks at 32°C. Mycolactone A/B was purified from bacterial pellets, as described elsewhere [6].

Patient cohort. A total of 37 patients with BUD were recruited by local health workers from villages near Tepa Government Hospital in the Ahafo Ano North District of Ghana, where there is a high prevalence of BUD (Table 1). Patients were recruited if they met the WHO clinical case definition of *M. ulcerans* disease; were not pregnant; were not receiving antibiotic treatment; had no history of tuberculosis, leprosy, or liver, kidney, or hearing impairment; and gave written informed consent (thumbprint of parent or guardian in the case of children, depending on literacy). Twenty-one healthy control participants were included from the same area of endemicity. In addition, 8 patients with pulmonary tuberculosis who had been receiving antimycobacterial treatment for 2–12 weeks were recruited from the Chest Clinic at Komfo Anokye Teaching Hospital, Kumasi, Ghana. The study protocol was approved by the ethics review committees at the School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, and St George's Hospital in London.

Diagnosis and treatment. Punch biopsy specimens 4 mm in diameter were obtained to confirm the clinical diagnosis, and treatment was started with 10 mg/kg oral rifampicin and 15 mg/kg intramuscular streptomycin daily, administered at village health posts under direct observation. If the diagnosis was confirmed by PCR for the IS2404 repeat sequence characteristic of *M. ulcerans,* antibiotic treatment was continued for 8 weeks. Biopsy specimens were also stained for acid-fast bacilli and cultured on Lowenstein-Jensen slopes, as described else-

where [22]. All lesions had healed by 6 months after the end of treatment, and there were no recurrences during the 10 months of posttreatment follow-up.

Whole-blood assay. Blood samples were obtained at 0, 4, 8, 12, and 48 weeks after the start of treatment for stimulation assays. Venous blood (12 mL) was obtained in sodium heparin Vacutainer tubes (Becton Dickinson). One-milliliter aliquots of undiluted blood were distributed in duplicate in 24-well tissue culture plates (Becton Dickinson) and incubated at 37°C in 5% CO_2 for 24 h in the presence of 10 μg/mL phytohemagglutinin (PHA) or no stimulant. After stimulation, supernatants were harvested and stored at −20°C until further analysis.

CD4$^+$ peripheral blood lymphocyte stimulation assay. Human peripheral blood lymphocytes were isolated from whole blood by sedimentation on a Ficoll-Hypaque gradient (GE Healthcare). CD4$^+$ T cells were then purified by negative selection (MACS; Miltenyi Biotec) and cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mmol/L L-glutamine (Sigma), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were plated in 96-well plates (105 cells/well) in the presence of increasing doses of mycolactone for 24 h and then stimulated for 24 h with 10 ng/mL phorbol-12-myristate 13-acetate (PMA) and 250 ng/mL calcium ionophore A23187. After stimulation, supernatants were harvested and stored at −20°C until further analysis.

Culture supernatant and serum analysis. Chemokines and cytokines were measured with a Luminex 100IS system (Milliplex MAP kits; Millipore), according to the manufacturer's instructions. In brief, 50 μL of biological fluid or standard was incubated with antibody-linked beads for 2 h, washed twice with wash solution, and incubated for 1 h with biotinylated secondary antibodies. A final incubation of 30 min with streptavidin-phycoerythrin preceded the acquisition on the Luminex system. At least 100 events were acquired for each analyte. Values above or below the standard curves were replaced by the lowest or the highest concentrations measured. Where applicable, 2-tailed nonparametric comparisons (Mann-Whitney *U* test) were performed to calculate *P* values. Statistical analyses were performed with the OmniViz program (version 5.2).

RESULTS

Selective suppression of circulating chemokines in patients with BUD. Taking advantage of a multianalyte profiling technology, we compared the circulating levels of a panel of inflammatory cytokines and chemokines in patients with BUD and healthy control participants from the same endemic zone. The serum levels of epidermal and vascular endothelial growth factor, intercellular and vascular adhesion molecules, and the metalloproteinases MMP2 and MMP9 were also examined, because they are potential indicators of tissue inflammation. Despite the presence of proliferating bacteria and massive tissue destruction in cutaneous lesions, the circulating levels of most of the studied analytes were not augmented in patients with BUD, compared with control participants (Figure 1). In fact, the 4 inflammatory chemokines macrophage inflammatory protein (MIP) 1β, IL-8, monocyte chemoattractant protein (MCP) 1, and (to a lesser extent) fractalkine were significantly suppressed.

Importantly, down-modulation of MIP-1β, IL-8, and MCP-1 was induced at the nodular stage and persisted during the ulcerative stage of the disease (Figure 2*A*). To evaluate the extent to which down-regulation of MIP-1β, IL-8, and MCP-1 is specific to BUD, these chemokines were assessed in patients from the same area who had pulmonary tuberculosis. Although the level of MIP-1β was lower in patients with tuberculosis than in control participants, those patients, unlike patients with BUD, did not have significantly reduced levels of IL-8 and MCP-1, which suggests that the selective suppression of these 2 chemokines is not a common feature of mycobacterial infections (Figure 2*B*).

Resolution of BUD-associated immunosuppression after antibiotic therapy. We next examined the dynamics of circulating chemokines during the course of antibiotic treatment. To gain insight into the host cellular responses, we also investigated a panel of T cell–derived cytokines. Serum samples were obtained at monthly intervals from patients receiving the antibiotic combination rifampicin-streptomycin for 8 weeks and after 10 months of posttreatment follow-up, and they were submitted to multianalyte profiling. As shown in Figure 3, this analysis demonstrated that inflammatory cytokines and chemokines were either not modulated or positively regulated during the course of antibiotic therapy.

Among the above-mentioned chemokines found to be suppressed in patients with BUD, we observed a marked augmentation of IL-8 and MCP-1 circulating levels 10 months after completion of antibiotic therapy, with concentrations reaching those of control participants (Figure 3*A*). A similar evolution was observed for MIP-1α, MIP-β, MCP-3, eotaxin, and fractalkine, although these data were more variable. In contrast, chemokines that were not suppressed in patients with BUD, such as IFN-inducible protein 10 or macrophage-derived chemokine, were not modulated during treatment.

With regard to T cell cytokines, only IL-6, IL-10, tumor necrosis factor (TNF) α, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were detectable in patient serum (Figure 3*B*). Whereas the circulating levels of IL-6, IL-10, and TNF-α were not modulated by antibiotic therapy, those of IL-4 and GM-CSF were significantly up-regulated 10 months after the end of antibiotic treatment (Figure 3*B*). Notably, in the case of IL-4, the serum levels are significantly higher than those of endemic control participants 10 months after com-

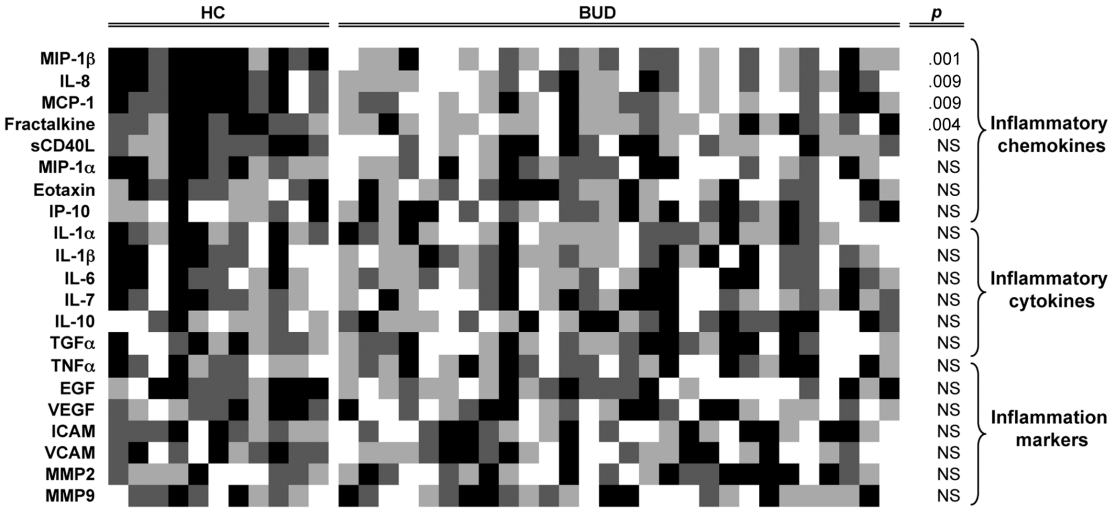

Figure 1. Selective suppression of circulating chemokines in patients with Buruli ulcer disease (BUD). Serum concentrations of a panel of inflammatory cytokines, chemokines, and inflammation markers were measured in patients with BUD ($n = 24$) and healthy control participants (HC) ($n = 11$), by use of Luminex technology. All values were normalized, and a heat map was used to mark quartile ranks for the entire data set. To identify induced or suppressed analytes, a Mann-Whitney *U* test was used, with a false discovery rate correction for multiple analyte testing ($P \leqslant .05$; NS, not significant). Findings were compared between patients with BUD and age- and sex-matched healthy donors from the region where disease was endemic. Data are mean values for duplicate wells. EGF, endothelial growth factor; ICAM, intercellular adhesion molecule; IL, interleukin; IP, interferon γ–inducible protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, metalloproteinase; sCD40L, soluble CD40 ligand; TGF, transforming growth factor; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; VEGF, vascular EGF.

pletion of the antibiotic therapy. The augmentation of IL-4 and GM-CSF coincided with that of the lymphoid survival factor IL-7 (Figure 3*B*).

Selective suppression of T cell functional properties in patients with BUD. Defective systemic production of IFN-γ has been reported in patients with BUD who have progressive ulcers, as evidenced by ex vivo stimulation of peripheral blood lymphocytes with mitogenic agents. Having shown that patients with BUD display a distinctive profile of immune suppression, we analyzed in depth the functionality of T cell populations in these patients. Supernatants of PHA-stimulated whole-blood cultures were submitted to a broad screen of cytokines and chemokines by means of the above-described multiplex technology. In accordance with other reports, stimulation-induced production of IFN-γ by Th1 cells was reduced in patients with BUD compared with healthy control participants (Figure 4). Even more dramatic was the suppression of IL-2, a critical T cell growth factor.

Th2 and Th17 responses were also down-modulated in patients with BUD, as indicated by the reduced levels of IL-4, IL-13, and IL-17 in cell supernatants. The capacity of T cells to produce GM-CSF on PHA stimulation was also suppressed in patients with BUD compared with control participants. A trend toward suppression was also observed for IL-10, although it was not statistically significant. Interestingly, this inhibitory effect was not observed with all T cell–derived cytokines; the production of IL-6 and TNF-α was comparable in the 2 groups. Overall, the results of this analysis suggested that the functional biology of all Th1, Th2, and Th17 cell subsets was impaired in patients with BUD, with a selective effect on the cytokines IL-2, IFN-γ, IL-4, IL-13, IL-17, and GM-CSF.

Immunological defects in BUD mimicked by action of mycolactone on CD4⁺ T lymphocytes. The data in Figures 1–4 identify a series of host inflammatory molecules that are systemically suppressed in patients with BUD. Among them are chemokines, which are expressed by all mononuclear cell subsets, and cytokines, which are primarily produced by $CD4^+$ T lymphocytes. We postulated that the defective cytokine and chemokine responses in patients with BUD may be caused by, at least partially, the immunosuppressive activity of mycolactone on this cell population. This possibility was investigated by incubating peripheral blood–derived $CD4^+$ T lymphocytes of healthy donors with increasing doses of the toxin and monitoring their cytokine and chemokine responses to stimulation.

For all of the inflammatory molecules tested, a marked and highly reproducible inhibitory action of mycolactone was observed, with nearly complete suppression of cytokine or chemokine production in the presence of 50 ng/mL mycolactone, when either PMA–calcium ionophore or antibodies to CD3 and CD28 were employed to stimulate activation (Figure 5*A* and data not shown). Importantly, a major proportion of the cells remained viable in the presence of biologically active concentrations of mycolactone (Figure 5*B*). With regard to IL-2 and IFN-γ, these results were confirmed independently by

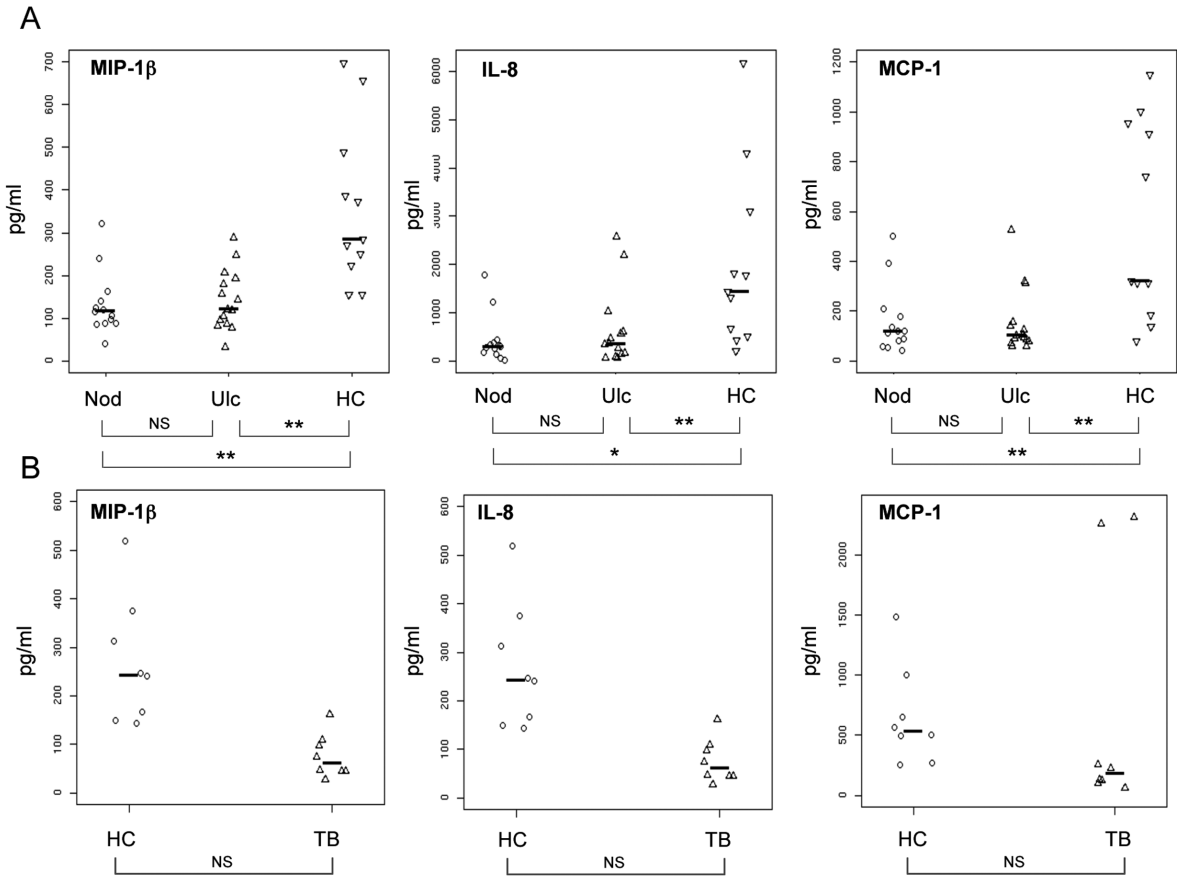

Figure 2. Down-modulation of macrophage inflammatory protein (MIP) 1β, interleukin (IL) 8, and monocyte chemoattractant protein (MCP) 1 induced at the nodular stage and specific for Buruli ulcer disease (BUD). *A*, Serum concentrations of MIP-1β, IL-8, and MCP-1, as measured by a Luminex system, were compared in 13 patients with BUD at the nodular stage (Nod), 15 patients with BUD at the ulcerative stage (Ulc), and 11 healthy control participants (HC). *B,* Serum concentrations of MIP-1β, IL-8, and MCP-1 in 8 patients with tuberculosis (TB) and 8 healthy control participants. Data are mean values of duplicate wells, which were analyzed with the OmniViz program. Mann-Whitney *U* tests were performed to obtain *P* values (*$P \leq .05$; **$P \leq .01$; NS, not significant). Each point corresponds to an individual donor, and lines indicate median values in each group. Data are representative of 2 independent experiments.

means of enzyme-linked immunosorbent assay (ELISA), with exposure to 20 ng/mL mycolactone inducing >75% suppression of cytokine production by $CD4^+$ T lymphocytes, irrespective of the stimulating agent (data not shown).

DISCUSSION

In some regions of sub-Saharan Africa, the incidence of BUD exceeds those of tuberculosis and leprosy, 2 major human infectious diseases that are caused by *M. tuberculosis* and *Mycobacterium leprae*, respectively. BUD contributes to a combined mycobacterial disease burden in impoverished rural communities, causing considerable morbidity and stigmatization [23]. A hallmark of these 3 pathogenic mycobacteria is their capacity to establish residence in the host and provoke long-term infections, for which they have evolved different strategies for evading and exploiting the host immune system. In particular, there is growing evidence that the unique lipids of their envelopes, at the interface between pathogen and host, play a central role in mycobacterial virulence. For example, the lipoarabinomannans and phosphatidylinositol mannosides of *M. tuberculosis* have been shown to modulate phagosomal maturation, thereby contributing to bacterial persistence in infected macrophages [24]. *M. tuberculosis* lipoarabinomannans and phenolic glycolipids also impair the production of proinflammatory mediators by professional antigen-presenting cells in vitro [25, 26]. As a potent suppressor of β-chemokine production by dendritic cells and inflammatory cytokine and chemokine production by monocytes, mycolactone therefore constitutes another type of mycobacterial lipid capable of inhibiting innate inflammatory responses.

The generation of specific cellular responses, particularly in the Th1 compartment, is crucial for protective immunity against most mycobacterial infections. In BUD, although the spontaneous healing of skin lesions usually coincides with a

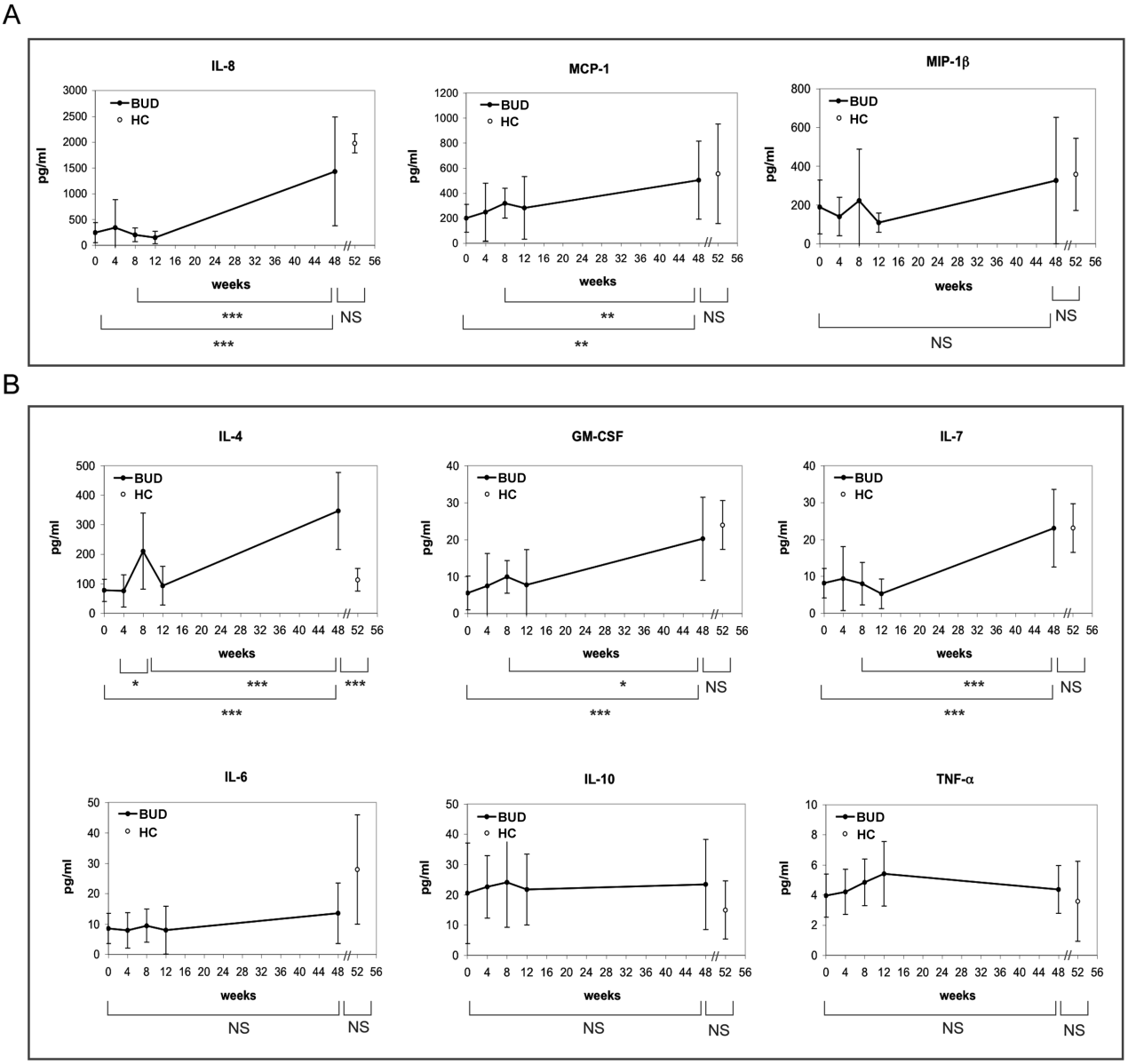

Figure 3. Resolution of Buruli ulcer disease (BUD)–associated immunosuppression after antibiotic therapy. The kinetics of serum concentrations are shown for a panel of inflammatory chemokines (*A*) and cytokines (*B*) in patients with BUD who were undergoing antibiotic treatment ($n = 9$). Data are means and standard deviations of duplicate Luminex measurements, which were performed at the beginning of therapy (0 weeks), after 4 weeks of treatment (4 weeks), at the end of the 8-week treatment (8 weeks), and 1 and 10 months after the end of treatment (12 and 48 weeks, respectively). Values were compared by means of repeated-measures analysis of variance with the Bonferroni post hoc test (*$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; NS, not significant). Data are representative of 2 independent experiments. GM-CSF, granulocyte-macrophage colony-stimulating factor; HC, healthy control participants; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

conversion to positive delayed-type hypersensitivity reaction against *M. ulcerans* antigens, the mechanisms involved in immune protection remain largely unknown [27]. A distinctive feature of BUD lesions is the lack of inflammation despite massive infection and tissue necrosis [28]. Patients who have BUD with active ulcers also show reduced systemic lymphoproliferative and IFN-γ responses [1]. Notably, these defects are not specific to mycobacterial antigens. Furthermore, they resolve after surgical excision of the lesions and curative antibiotic therapy [29], strongly suggesting that a bacterial factor inhibits cellular responses locally and systemically. Here we show that, in fact, the systemic suppression of cellular responses in BUD affects not only the Th1 cell compartment but also the Th2 and Th17 cell subsets. This defect is not due to differences in T cell concentrations, because blood cell counts were comparable in patients with BUD and in control participants

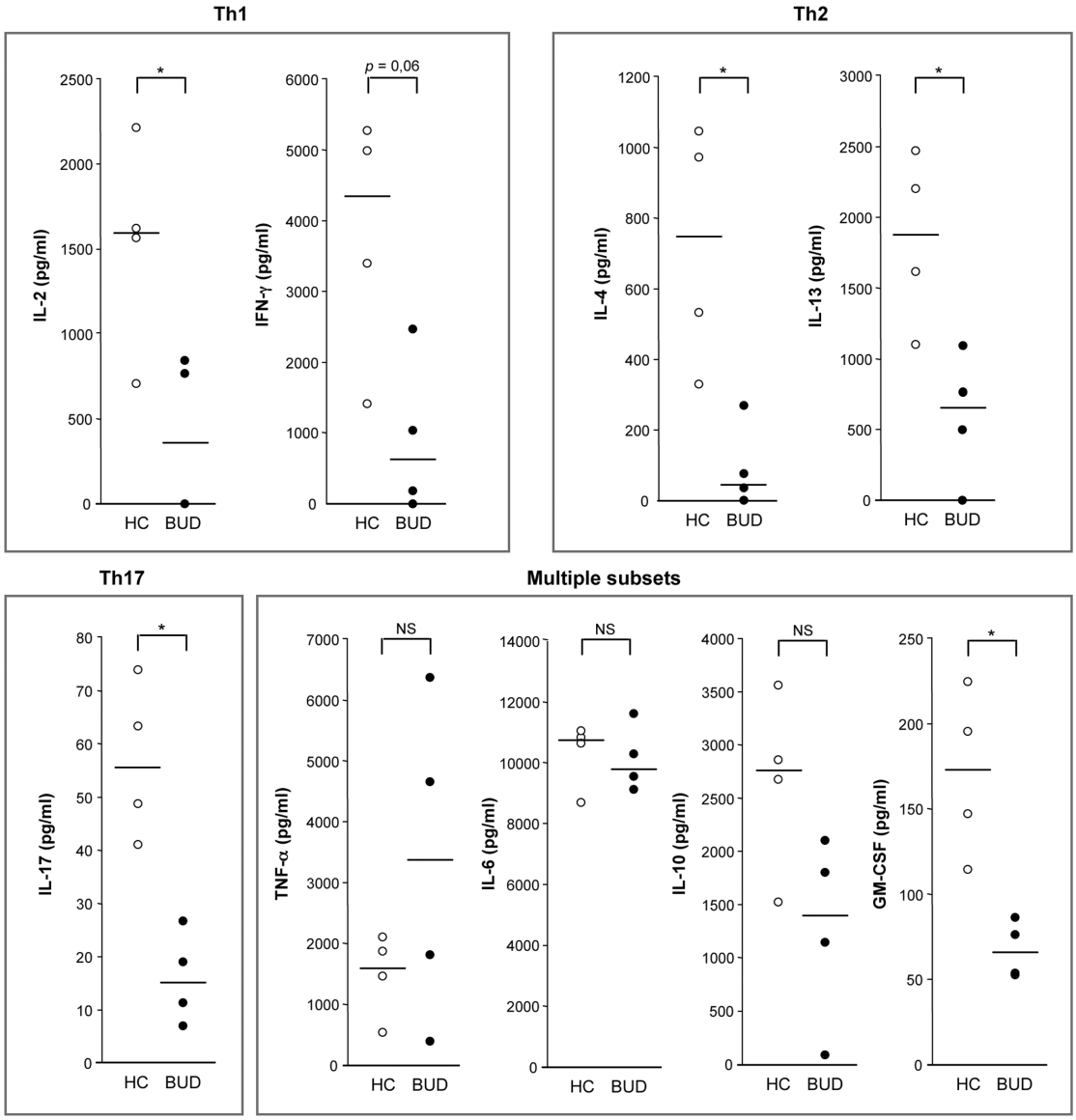

Figure 4. Selective suppression of T cell functional properties in patients with Buruli ulcer disease (BUD). For 4 patients with BUD and 4 healthy control participants (HC), T cell–derived cytokines were assessed with a Luminex system in supernatants of whole-blood stimulation assays. Data correspond to mean values of phytohemagglutinin-stimulated duplicate wells, from which the mean value of unstimulated control wells was subtracted. Data were analyzed with the OmniViz program, and Mann-Whitney *U* tests were performed to obtain *P* values (*$P \leqslant .05$; NS, not significant). Each point corresponds to an individual donor, and lines indicate median values in each group. Data are representative of 2 independent experiments. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

(R. Phillips, unpublished data). In accordance with other reports, these findings demonstrate that defective T cell responsiveness to stimulation is not antigen specific but is rather associated with a functional blockade of T cell capacity to express, or secrete, inflammatory products. Because suppression of T cell functions occurs without loss of cell viability (Figure 5), it may be the result not of a cytotoxic action of mycolactone but rather of the induction of an anergic state, the mechanism of which remains to be investigated.

In the face of such a broad and antigen-independent suppression of T cell responses, it is unclear why patients with BUD are not more susceptible to secondary infections. Although recently an increased prevalence of human immunodeficiency virus (HIV) infection was reported in patients with BUD in Benin, the frequency of superinfections remains surprisingly low, and patients with BUD typically show a good general health status other than the progression of skin lesions [30]. This may be partially explained by the fact that systemic suppression of cellular responses in patients with BUD is incomplete and reversible. Although Th1 responses are signifi-

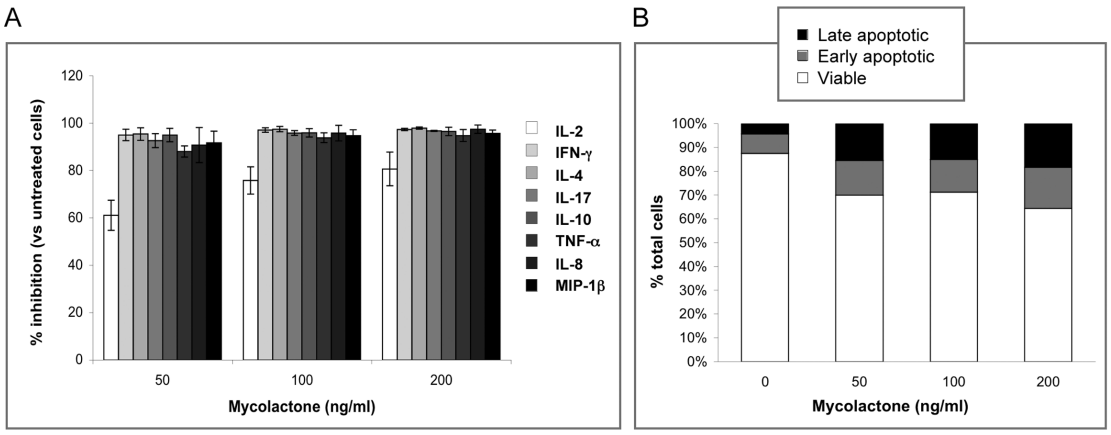

Figure 5. Defective cytokine and chemokine responses in patients with Buruli ulcer disease mimicked by action of mycolactone on $CD4^+$ T lymphocytes. *A,* Suppression of interleukin (IL) 2, interferon (IFN) γ, tumor necrosis factor (TNF) α, IL-4, IL-10, IL-17, macrophage inflammatory protein (MIP) 1β, and IL-8 production by human $CD4^+$ peripheral blood lymphocytes. Cells incubated with mycolactone for 24 h before stimulation with phorbol-12-myristate 13-acetate and calcium ionophore for 24 h are compared with cells stimulated in the absence of mycolactone. Data are means and standard deviations of duplicate measurements obtained with a Luminex system in 3 independent donors. *B,* Effect of 90-h incubation with mycolactone on cell viability, as measured by a flow cytometric approach using propidium iodide (PI) and fluorescein isothiocyanate–labeled annexin V staining. Annexin V^+/PI^- cells were identified as early apoptotic cells, annexin V^+/PI^+ cells as late apoptotic cells, and annexin V^-/PI^- cells as live cells. Data are representative of 2 independent experiments.

cantly impaired in patients with BUD, particularly during the early stages of the disease, host T cells remain viable and functional as the disease progresses, as evidenced by the vigorous inflammation occurring in the skin after antibiotic administration [31–33]. The success of *M. ulcerans* in the establishment of a persistent infection may thus be viewed as a balance between pathogen and host, in which mycolactone suppresses but does not neutralize the development of host immune responses.

The relevance of lymphocyte immunosuppression in the context of a natural infection with *M. ulcerans* relies on the pharmacodistribution of the toxin and its ability to interact with target cells in the host. Although hematogenous dissemination of *M. ulcerans* has been reported, the fact that bacteria remain essentially localized in subcutaneous tissue raises the question of how mycolactone gains access to lymphocytes in vivo [14]. Once mycobacteria are phagocytosed by host cells, their lipid constituents are thought to traffic within subcellular compartments and be released by infected cells via the formation of vesicles [34]. Cells harboring intracellular *M. ulcerans*, or lesions containing extracellular bacteria, may similarly release the toxin to the medium. This is in accordance with the unusual histopathological feature of Buruli ulcers, which suggests a radial diffusion of mycolactone from the infectious foci. In addition, we have evidence from mouse studies that mycolactone produced in peripheral tissues can reach internal organs, because structurally intact mycolactone was identified in blood and spleen lymphocytes of mice subcutaneously injected with mycolactone or infected with *M. ulcerans* [7]. These data, therefore, are consistent with the notion that mycolactone permits *M. ulcerans* to establish long-term infections by remotely neutralizing the development of cellular responses.

BUD is usually diagnosed on the basis of clinical findings, because laboratory tests based on smear examination, *M. ulcerans* cultures, or PCR detection require significant logistics and equipment. Alternative diagnostic approaches are urgently needed to allow the early identification of patients and enable more effective treatment. In this study, we identified a series of immunological mediators, the production of which is systemically suppressed in patients with BUD. Using an algorithm based on the serum levels of MIP-1β, IL-8, MCP-1, and fractalkine, we were able to identify correctly 100% of the patients with BUD and 64% of the control participants (data not shown). In basal conditions, MIP-1β, IL-8, and MCP-1 are present in patient serum in significant amounts. They are directly and easily assessable with simple, inexpensive, and field-friendly assays, such as ELISAs. Furthermore, our preliminary investigations show that suppression of IL-8 and MCP-1 is induced early in BUD. To our knowledge, this is the first report of a down-modulation of these chemokines in association with an infectious disease. Although a follow-up cohort would be required to validate these findings for clinical use, the findings of this study strongly suggest that measuring the levels of MIP-1β, IL-8, and MCP-1 in serum may help confirm a clinical diagnosis of BUD.

Until recently, the only available treatment for BUD was surgical excision of the lesion. Today, WHO recommends a daily administration of rifampicin (oral) and streptomycin (intramuscular) for 8 weeks, treatment that represents consider-

able progress in the management of the disease. To optimize the frequency, duration, and route of administration of antibiotics, sensitive biomarkers of the therapeutic response of patients are now required. Our analysis of patient serum during the course of antibiotic therapy showed that most of the BUD immunological correlates were positively modulated by antibiotic therapy. However, their up-regulation was a slow process, beginning after completion of the treatment. In the absence of reagents to detect mycolactone, we cannot determine whether this reflects the progressive elimination of mycolactone, or other mycobacterial products, from the host organism. Notably, IL-4 was the only inflammatory marker to be modulated during the course of antibiotic therapy, with serum levels increasing significantly during the period between 4 and 8 weeks of treatment. The stimulation of IL-4 production was intense, with circulating levels reaching higher concentrations than those of control participants 10 months after the end of antibiotic treatment. IL-4 is a key T cell cytokine that stimulates both activated B and T cell proliferation and the differentiation of $CD4^+$ T cells into Th2 cells. The administration of antibiotics often leads to an active inflammatory process at the site of infection, with massive infiltration of activated T and B cells [34]. The up-regulation of IL-4 in patient serum may thus reflect the reversal of mycolactone-induced local immunosuppression and restoration of active humoral and cellular immune responses, thereby constituting a valuable tool for monitoring patient responses to antibiotic treatment.

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