Systemic suppression of interferon-γ responses in Buruli ulcer patients resolves after surgical excision of the lesions caused by the extracellular pathogen *Mycobacterium ulcerans*

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Abstract: Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is the third most common mycobacterial infection in immunocompetent humans besides tuberculosis and leprosy. We have compared by ex vivo enzyme-linked immunospot analysis interferon-γ (IFN-γ) responses in peripheral blood mononuclear cells (PBMC) from BU patients, household contacts, and individuals living in an adjacent *M. ulcerans* nonendemic region. PBMC were stimulated with purified protein derivative (PPD) and nonmycobacterial antigens such as reconstituted influenza virus particles and isopentenyl-pyrophosphate. With all three antigens, the number of IFN-γ spot-forming units was reduced significantly in BU patients compared with the controls from a nonendemic area. This demonstrates for the first time that *M. ulcerans* infection-associated systemic reduction in IFN-γ responses is not confined to stimulation with live or dead mycobacteria and their products but extends to other antigens. Interleukin (IL)-12 secretion by PPD-stimulated PBMC was not reduced in BU patients, indicating that reduction in IFN-γ responses was not caused by diminished IL-12 production. Several months after surgical excision of BU lesions, IFN-γ responses of BU patients against all antigens used for stimulation recovered significantly, indicating that the measured systemic immunosuppression was not the consequence of a genetic defect in T cell function predisposing for BU but is rather related to the presence of *M. ulcerans* bacteria. *J. Leukoc. Biol.* 79: 1150–1156; 2006.

Key Words: *ex vivo ELISpot analysis · immunosuppression · interleukin-12*

INTRODUCTION

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is an infectious disease characterized by chronic, necrotizing ulceration of subcutaneous (s.c.) tissues and the overlying skin. The disease starts as a s.c. nodule, papule, or plaque, which eventually ulcerates and progresses over weeks to months until surgical excision or spontaneous healing occurs [1]. After tuberculosis and leprosy, BU is the third most common mycobacterial infection in immunocompetent humans [2]. The main burden of disease falls on children living in sub-Saharan Africa, but healthy people of all ages, races, and socioeconomic class are susceptible [3]. The effectiveness of antimycobacterial drug therapy has not been proven [3]. Consequently, surgery is presently the recommended treatment option [4]. In BU lesions, clumps of extracellular, acid-fast organisms surrounded by areas of necrosis are found, particularly in s.c. fat tissue [5]. *M. ulcerans* produces a family of macrolide toxin molecules, the mycolactones, which are associated with tissue destruction and local immunosuppression [6]. In cell culture experiments, mycolactones produce apoptosis and necrosis in many human cell types [7, 8]. The toxin appears to play a role in inhibiting the recruitment of inflammatory cells to the site of infection, which explains at least in part why inflammatory responses are poor in BU lesions [5]. However, intralesional influx of leukocytes and granulomatous responses in the dermis and panniculus has been reported in late stages of the disease [9, 10]. Spontaneous healing can occur and is often accompanied by a conversion of the Burulin (*M. ulcerans* sonicate) skin test from negative to positive. However, the immune mechanisms involved in protection against BU are largely unknown.

The importance of interferon-γ (IFN-γ) for immunity against mycobacterial infections in humans is demonstrated by the increased susceptibility of children carrying complete IFN-γ receptor 1 (IFN-γR1) chain deficiency to environmental mycobacterial infection [11]. Apart from $CD4^+$ T cells, γδ T cells, natural killer cells (NK), and $CD8^+$ T cells are potent sources of IFN-γ. CD4 T cells can be differentiated into T helper cell

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type 1 (Th1) and Th2, distinguished by their patterns of cytokine production after antigen activation. Apart from other cytokines, Th1 cells preferentially secrete IFN-γ, and Th2 cells preferentially secrete interleukin (IL)-4 and IL-5. Th1 or Th2 development is determined by the cytokine environment during T cell activation in the primary response to antigen, and IL-12 and IFN-γ are implicated in the decision to adopt a Th1 phenotype [12]. IFN-γ binds to the IFN-γR1/IFN-γR2 complex and stimulates innate cell-mediated immunity through NK cells and activation of bactericidal mechanisms in macrophages. The central role of IFN-γ in major histocompatibility complex class I- and class II-restricted antigen processing and presentation is well-documented [13]. At present, the contribution of IFN-γ in immunity to extracellular *M. ulcerans* remains to be established.

Peripheral blood mononuclear cells (PBMC) of BU patients with active disease showed significantly reduced lymphoproliferation and IFN-γ secretion in response to stimulation with live or killed preparations of *Mycobacterium bovis*, *M. ulcerans*, *M. tuberculosis*, and the recombinant protein Ag85 of *M. tuberculosis* [14–18]. Here, we have determined in a cross-sectional study the frequency of IFN-γ-secreting cells in PBMC from BU patients, their household contacts, and controls from BU nonendemic areas by ex vivo enzyme-linked immunospot (ELISpot) analysis. The antigens used for stimulation included isopentenyl pyrophosphate (IPP), reconstituted influenza virus particles (virosomes), and tuberculin-purified protein derivative (PPD) of *M. tuberculosis*, which stimulate distinct T cell subsets, such as Vγ2Vδ2 T cells [19], CD4 T cells [20], and CD4 and Vγ2Vδ2 T cells [21], respectively. Results demonstrate that BU-associated reductions in IFN-γ responses are not confined to stimulation with live or dead mycobacteria and mycobacterial antigens. Furthermore, it is shown for the first time that in individual BU patients, this suppression in IFN-γ secretion improved in a time interval of 5–10 months.

MATERIALS AND METHODS

Study population

Thirteen BU patients, 19 clinically healthy household contacts who never had clinical BU, and 18 healthy persons living in a *M. ulcerans* nonendemic district in the Greater Accra region of Ghana were enrolled for the study (**Table 1**). All BU patients enrolled were residents of the BU-endemic Ga District and presented with preulcerative or ulcerative lesions at the Amasaman Health Centre. Informed consent was obtained from study participants or their parents or guardians before enrollment. For ethical reasons, the age range of the nonexposed controls, who were included into the analysis, was higher than that of the BU patients. Ethical approval for the study was obtained from the local ethical review board of the Noguchi Memorial Institute for Medical Research, University of Ghana (Legon). Clinical diagnosis of BU was reconfirmed as described [22, 23] by one or more laboratory verification tests (**Table 2**), including culture of *M. ulcerans*, microscopic detection of acid-fast bacilli (AFB), or IS*2404* polymerase chain reaction (PCR). The clinical pictures of the patients ranged from nodules or plaques to severe ulcerative forms (Table 2). In the BU group, nine persons and in the other two groups, 10 persons each with a BCG scar were recruited (Table 1). PBMC were isolated from venous peripheral blood using Ficoll-Hypaque gradient centrifugation following standard procedures and cryopreserved prior to the analysis.

Antigens

After thawing, PBMC were directly stimulated with 50 μM IPP (Sigma Chemical Co., St. Louis, MO), 10 μg/ml tuberculin PPD derivative of *M. tuberculosis* (Statens Seruminstitut, Denmark), 10 μg/ml phytohemagglutinin (PHA; Sigma Chemical Co.), or 20 μg/ml immunopotentiating reconstituted influenza virosomes (IRIV; Berna Biotech, Switzerland). These influenza virosomes are spherical, unilamellar vesicles prepared by detergent removal from influenza surface glycoproteins and mixtures of natural and synthetic phospholipids containing H1N1 from influenza virus strain A/Singapore/6/86 [24]. Cell culture medium consisted of RPMI 1640, 10% heat-inactivated human AB serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, Grand Island, NY).

Ex vivo IFN-γ ELISpot analysis

PBMC were thawed, washed, and suspended at a concentration of 2×10^6 cells/ml in complete cell culture medium. The cells were then stimulated with the different antigens at the final concentrations indicated above and incubated for 24 h at 37°C, 5% CO_2 humidified atmosphere. A 96-well nitrocellulose-bottomed plate (Millipore, Beford, MA) was coated overnight at 4°C with 10 μg/ml anti-human IFN-γ primary antibody (Clone1-D1K; Mabtech, Sweden). The plates were then washed five times with phosphate-buffered saline (PBS) and blocked with cell culture medium for 1 h at room temperature. The medium was decanted, and preincubated cells (2×10^5 or 1×10^5) were added to each well in triplicate and incubated at 37°C for another 20 h. Assays were terminated by washing plates three times with PBS-Tween 80 (0.05%) followed by PBS another three times. Secondary antibody (biotin-labeled anti-human-IFN-γ; Clone 7-B6-1) was added to each well at 1 μg/ml, and the plate was incubated for 2 h at room temperature (RT). Plates were washed again six times with PBS before application of streptavidine-alkaline phosphatase (1:1000 dilution in PBS, 0.5% fetal calf serum, 100 μl/well) for 1 h at RT. After washing wells seven times with PBS, distinct spots were developed by the incubation of plates at RT for 4–10 min following the addition of the developing buffer (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, diluted 1:100, Bio-Rad, Hercules, CA). Spot development was stopped by washing the plates extensively with water and left to dry. Plates were later evaluated using the ELISpot Reader system (AID, Germany) to determine the number of spot-forming units (SFU).

TABLE 1. Characteristics of BU Patients, Household Contacts, and Controls from a BU Nonendemic Area Enrolled in the Study

Characteristic	BU patient (n = 13)	Household contacts (n = 19)	Controls from nonendemic area (n = 18)
Sex			
Male	5 (39%)	6 (32%)	8 (44%)
Female	8 (61%)	13 (68%)	10 (56%)
Median age (years)	15	15	29.5
Age range (years)	6–45	6–56	24–65
BCG scar present (%)[a]	69	52	55

[a] Percent values calculated for 13 patients, 14 household contacts, and 14 controls, respectively. Five household contacts and four nonexposed controls remained with uncertain scar status. BCG, Bacillus Calmette-Guerin.

TABLE 2. Clinical Data of Patients Presenting with Lesions As a Result of Confirmed *M. ulcerans* Infection

Patient	Age	Gender	Clinical form[a]	BCG status[b]	AFB[c]	Culture[d]	PCR[e]	First blood sample[f]	Second blood sample[f]
P03	45	f	ulcerative	–	+	+	+	3 months	11 months
P04	9	f	plaque	+	–	+	+	0	8 months
P05	10	m	nodule	+	+	+	+	0	8 months
P08	16	m	ulcerative	–	+	+	+	4 months	14 months
P09	5	f	ulcerative	+	+	–	+	3 months	12 months
P11	20	f	ulcerative	+	+	+	+	7 days	7 months
P12	16	m	ulcerative	+	–	–	+	3 months	12 months
P13	11	f	ulcerative	–	+	+	+	7 days	7 months
P14	15	m	plaque	–	–	–	+	7 days	8 months
P15	7	f	ulcerative	+	+	+	+	0	9 months
P19	6	m	nodule	+	+	+	+	0	6 months
P21	17	f	nodule	+	+	–	+	0	5 months
P22	17	f	nodule	+	+	+	–	0	5 months

[a] Clinical forms of BU disease were graded according to the World Health Organization case definition [1]. [b] BCG status was determined by confirmation of the presence of a BCG scar by two persons. [c] AFB detection was performed by direct smearing of tissue exudates followed by Ziehl-Neelsen staining [1]. [d] Culture of *M. ulcerans* according to ref. [22]. [e] IS*2404* PCR analysis according to ref. [23]. [f] Time of blood sample collection. Time 0 is time of surgical treatment.

Enzyme-linked immunosorbent assay (ELISA) quantification of IL-12 in cell culture supernatants

Levels of total IL-12 in culture supernatant of PBMC incubated with PHA (10 μg/ml) and PPD (10 μg/ml) for 96 h were determined by ELISA using a commercial kit (Mabtech). Samples were analyzed in triplicates, and results were expressed as the average of the three readings in an ELISA reader at 450 nm with reference to curves generated using serially diluted recombinant human IL-12. The sensitivity of the assay was 30 pg/ml for total IL-12.

Statistical analysis

Data were analyzed using the STATA program (Stata Corporation, College Station, TX). Comparisons among the paired samples were performed using the Wilcoxon signed-rank test, and the Wilcoxon rank-sum test was used to analyze the significance of observed difference in IL-12 secretion between patients and unexposed controls. For comparing the frequencies of antigen-specific IFN-γ-secreting cells, the data were transformed to normality using Box-Cox transformation before being analyzed by linear regression. Data were considered statistically significant when $P < 0.05$.

RESULTS

Frequency reduction of IFN-γ-secreting SFU in PBMC from BU patients

Thirteen patients with laboratory-reconfirmed *M. ulcerans* infection, 19 clinically healthy household contacts, and 18 individuals from a neighboring BU nonendemic area were enrolled for this study (Tables 1 and 2). The frequency of immediate IFN-γ-secreting cells in PBMC upon stimulation with IPP, IRIV, and PPD was analyzed using an ex vivo ELISpot assay. **Figure 1** shows that the mean of SFU upon stimulation with PPD, IPP, and IRIV was significantly lower ($P=0.0086$, $P=0.001$, and $P=0.0002$, respectively) in BU patients compared with nonexposed controls. In household contacts, the mean of SFU after IPP and IRIV stimulation was significantly higher compared with BU patients ($P=0.005$ and $P=0.001$,

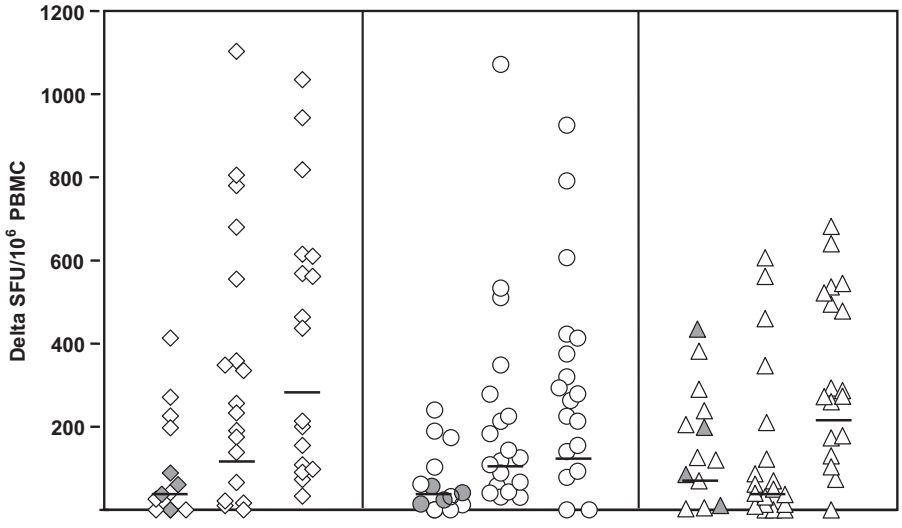

Fig. 1. Quantification of ex vivo IFN-γ-secreting cells by ELISpot analysis after stimulation of PBMC with IPP (◇), IRIV (○), and PPD (Δ). PBMC derived from BU patients, household contacts, and controls from a BU nonendemic area were thawed into cell culture medium and kept overnight in the presence or absence of the different stimuli. For the detection of IFN-γ-producing cells, 2×10^5 PBMC/well were plated in triplicate wells, and spots were developed after 24 h. Delta SFU = Mean of SFU of stimulated triplicate cultures – mean SFU of unstimulated triplicate cultures. Data from each individual analyzed are shown separately. Mean SFU/10^6 PBMC were 26 (6–102), 137 (57–327), and 262 (157–436) after stimulation with IPP in BU patients, household contacts, and nonendemic controls, respectively. In IRIV-stimulated cells, the mean SFU/10^6 cells were 22 (7–70), 135 (83–221), and 149 (56–394) in BU patients, household contacts, and nonendemic controls, respectively. After PPD stimulation, 88 (35–223), 39 (14–106), and 212 [100–445] SFU/10^6 PBMC were recorded in BU patients, household contacts, and nonendemic controls, respectively. The values given in brackets are the 95% confidence intervals of the geometric means of SFU. Data of Patients P03, P08, P09, and P12 are shown by shaded symbols.

respectively), and in PPD-stimulated cultures, no significant difference was observed ($P=0.82$).

Recovery from systemic immunosuppression after surgical treatment

All patients enrolled for the study were treated by wide surgical excision of the BU lesions. Blood samples were collected several months after the first sampling (Table 2), and PBMC from both time-points were analyzed in parallel using the same IFN-γ ELISpot assay as above. A 3.9-, 3.7-, and 3.6-fold median increase in cellular responses against IPP, IRIV, and PPD stimulation, respectively, was observed when PBMC, taken at the two time-points, were compared (**Fig. 2A**). Statistical analysis confirmed this increase of responses as highly significant with P values of 0.021 and 0.003, respectively, after IPP and IRIV stimulation. PHA-stimulated control wells showed only a slight (1.7-fold) median increase between the two different time-points analyzed ($P=0.17$), and in PPD-stimulated cultures, the boost was not significant ($P=0.09$). One representative example of an ELISpot analysis is shown in Figure 2B, and the numbers of SFU detectable after IPP, IRIV, and PPD stimulation rose between the first and second sampling. In contrast, medium and PHA control wells remained at a comparable level between the two analyses.

IL-12 production in PPD-stimulated PBMC is not affected in BU patients

Next, we wanted to determine whether the systemic suppression of IFN-γ responses in BU patients is related to a diminished capacity to secrete IL-12. PBMC of nine patients and 10

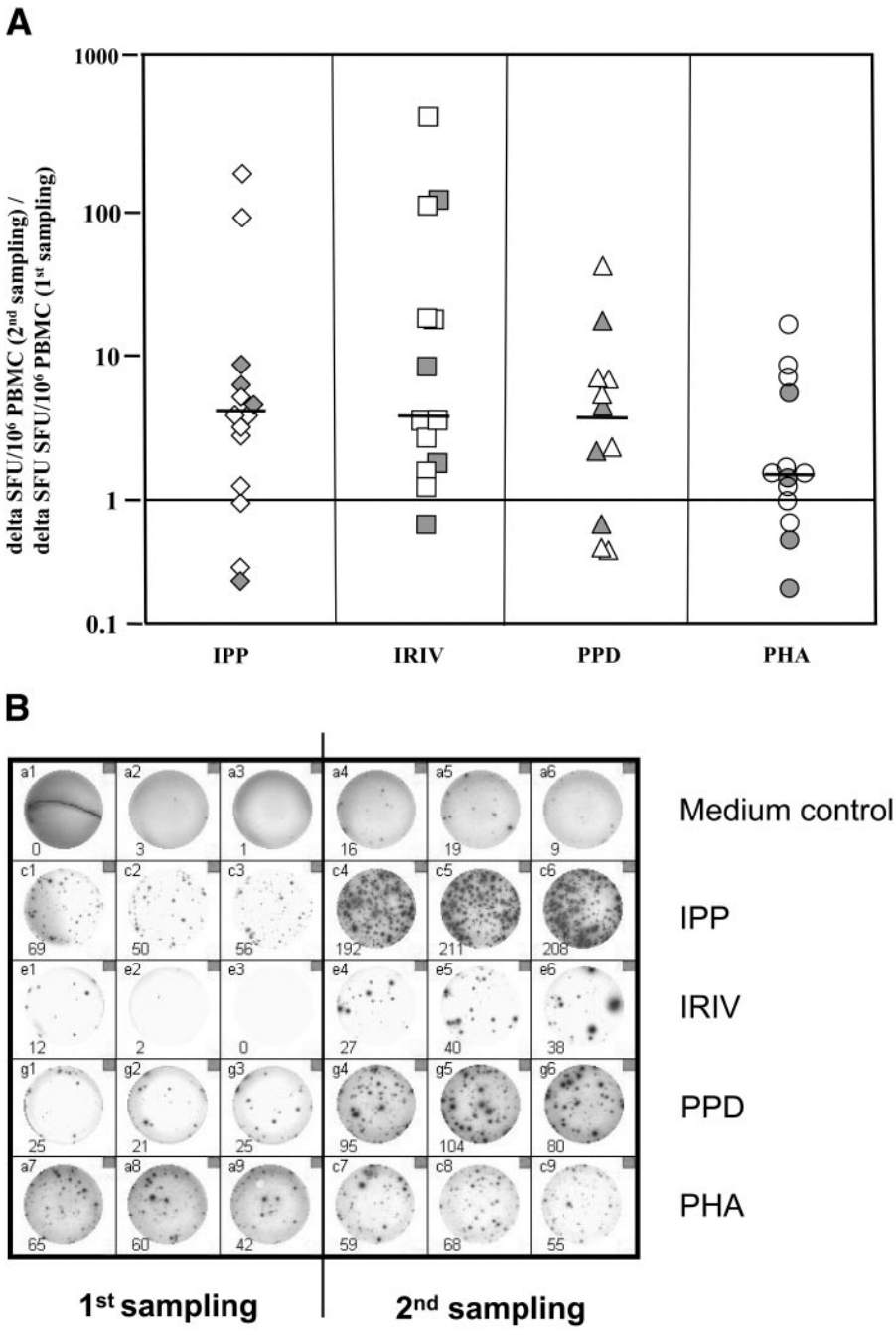

Fig. 2. (A) Frequency increase of antigen-specific ex vivo IFN-γ-secreting cells in BU patients. PBMC of 13 BU patients sampled at two time-points (Table 2) were thawed into cell culture medium and kept overnight in the presence or absence of IPP (◇), IRIV (□), PPD (Δ), and PHA (○). For the detection of IFN-γ-producing cells, 2×10^5 PBMC/well were plated in triplicate wells, and spots were developed after 24 h. Delta SFU were calculated as described in Figure 1. Given is the ratio of SFU obtained after the second sampling/first sampling. Data of Patients P03, P08, P09, and P12 are shown by shaded symbols. (B) Representative IFN-γ ELISpot patterns of BU Patient P21. PBMC of BU Patient P21 were obtained immediately before surgical excision of the BU lesion (first sampling) and 5 months later (second sampling). Parallel analysis of both samples was conducted in triplicates arranged horizontally with 2×10^5 cells/well plated. The different stimulators used are given on the right, and the numbers of SFU/well are shown in the lower-left corners.

persons from BU nonendemic areas were stimulated with PHA or PPD, and the total IL-12 concentration in cell culture supernatants was measured by ELISA (**Fig. 3**). It is interesting that the IL-12 concentrations in PPD-stimulated PBMC of individuals living in *M. ulcerans* nonendemic regions were statistically lower compared with BU patients ($P=0.011$), and in PHA-stimulated cultures, no significant difference was observed ($P=0.57$; Fig. 3). The mean IL-12 concentrations in supernatants of PPD- and PHA-stimulated cultures of PBMC obtained before or after surgical treatment showed no difference (Fig. 3).

DISCUSSION

An ex vivo ELISpot assay for detection of IFN-γ secretion permitting the direct detection of individual antigen-specific T cells at low frequencies was used in the current study [25]. The 48-h antigen challenge of PBMC suffices to engage cytokine production in the memory/effector T lymphocyte but not in naïve T cells and allows determination of frequencies and cytokine signatures of recirculating, antigen-specific T cells [25]. The frequencies of systemic IFN-γ-producing SFU after stimulation with IPP, IRIV, or PPD were reduced significantly in BU patients compared with individuals living in a neighboring BU nonendemic area. This result is consistent with other reports demonstrating that PBMC from subjects with past or current *M. ulcerans* disease had reduced IFN-γ production in response to PPD of *M. tuberculosis* and *M. bovis* or whole-killed *M. bovis* BCG or *M. ulcerans* [14–18]. However, our data strongly indicate that reduced IFN-γ production is not confined to immune responses specific for mycobacterial antigens or whole mycobacteria but extends to CD4 T cell responses specific for influenza virus [20] and Vγ2Vδ2 T cells [19].

Reduced IFN-γ production in BU patients could be the consequence of a genetic abnormality in T cell function predisposing for mycobacterial infections [11]. Therefore, we analyzed PBMC of BU patients sampled at two consecutive time-points. When the paired PBMC samples were analyzed in parallel by ELISpot analysis, the numbers of IFN-γ-secreting cells after IPP, IRIV, and PPD stimulation increased significantly after surgical treatment. Comparable numbers of SFU in PHA-stimulated PBMC in paired samples excluded a general suppression of cellular immune responses in BU and variations in quality of PBMC sample cryopreservation. To our knowledge, this is the first report demonstrating that antigen-specific IFN-γ production in BU patients is coming back to normal levels after surgical treatment. Hence, confounding genetic defects do not seem to be responsible for the observed immunosuppression. The question of whether IFN-γ production in patients undergoing spontaneous healing of BU will also improve over time is not addressed here.

IL-12 induces T and NK cells to produce several cytokines, including IFN-γ [26]. Total IL-12 concentrations in culture supernatants of PPD-stimulated PBMC were statistically higher in BU patients (treated and untreated) than in the controls. This may reflect a compensatory mechanism of the immune system to restore IFN-γ production and indicates that the observed reduction in systemic IFN-γ responses is not caused by diminished IL-12 production. Different cytokine expression profiles in PBMC and skin lesions were described in patients suffering from the nodular and ulcerative forms of BU. Nodules were associated with higher IFN-γ and lower IL-10 production and BU, with lower IFN-γ and higher IL-10 production [17]. Within the limited number of BU patients enrolled in our study, a relationship between different stages of BU disease and reduction of IFN-γ secretion was not observed.

Vγ2Vδ2 T cells compose the majority of human γδ T cells in circulation and among their defined ligands, is IPP, a metabolite found in prokaryotic and eukaryotic cells including mycobacteria [27]. Studies in rhesus monkeys provided evidence that Vγ2Vδ2 T cells contribute to adaptive immune

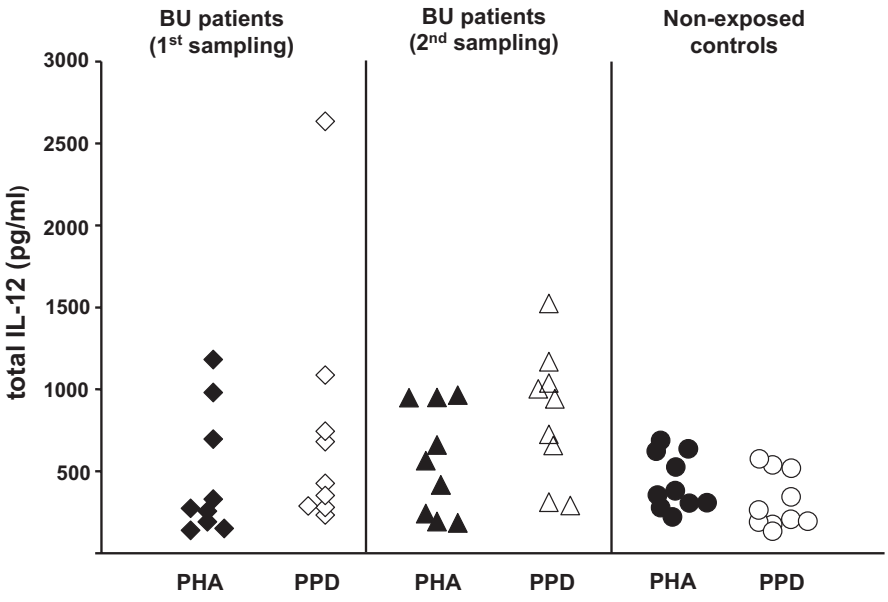

Fig. 3. Total IL-12 concentrations in cell culture supernatants of PHA- and PPD-stimulated PBMC of nine BU patients obtained at the two different sampling time-points (Table 2). Mean of total IL-12 concentration (given in pg/ml) in PPD-stimulated cultures was 543 and 750 after the first and second sampling, respectively, and in PHA-stimulated cultures, 348 and 472, respectively. PBMC of 10 individuals living in a neighboring *M. ulcerans* nonendemic region were treated similarly, and the mean total IL-12 concentrations after PPD and PHA stimulation were 276 and 402, respectively.

responses in mycobacterial infections [28]. A correlation between the absence or loss of Vγ2Vδ2 T cells and the extent of *M. tuberculosis* disease has been described [29]. An impaired ability of Vγ2Vδ2 T cells to produce cytokines or proliferate in response to phosphorylated microbial metabolites was observed in active *M. tuberculosis* pulmonary disease [30–32]. It is interesting that in 10 out of 13 BU patients analyzed here, the ex vivo IFN-γ secretion of IPP-reactive Vγ2Vδ2 T cells increased significantly after surgical treatment.

The diffusible macrolide toxins produced by *M. ulcerans* are considered as virulence factors responsible for pathogenicity of *M. ulcerans*, and it has been hypothesized that lack of inflammatory responses in BU lesions are related to local immunosuppressive activities of the mycolactones [6, 33, 34]. The treatment-associated reversal of immune suppression may indicate that mycolactones exert, apart from local, systemic effects also. Alternatively, additional, other immune-suppressive mechanisms may be operative in chronic *M. ulcerans* disease. PBMC from healthy contacts of tuberculosis patients and tuberculosis patients with limited disease produce large quantities of IFN-γ in response to whole mycobacteria, PPD of *M. tuberculosis*, early secretory antigenic target-6, and 16-kDa and 38-kDa proteins [35–39]. In contrast, PBMC of active tuberculosis patients with advanced disease produce low quantities of IFN-γ after similar stimulation, and following effective drug treatment, the IFN-γ secretion improved [39, 40]. Possible candidate structures mediating immune suppression in *M. tuberculosis* isolates such as phenolic glycolipids have been described [41, 42]. In leprosy induction of regulatory or suppressor T cells, mediating immune suppression in affected hosts has been proposed [43, 44]. Our findings, within their limits as a result of the small number of BU patients analyzed and the timing of the blood samples drawn, may indicate that mycolactone-independent immunosuppressive mechanisms common to chronic mycobacterial infections contribute to the reduction of systemic IFN-γ responses in BU patients.

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