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Leprosy Patients with Lepromatous Disease Have an Up-regulated IL-8 Response That Is Unlinked to TNF-α Responses[1]

Z. Hasan, A. Mahmood, S. Zafar, A. A. Khan, and R. Hussain[2]

ABSTRACT

Tumor necrosis factor (TNF-α) in conjunction with interferon-γ (IFN-γ) plays an important role in lymphocyte recruitment and granuloma formation in mycobacterial diseases. Lepromatous leprosy infections are typically associated with low to absent T cell responses and the absence of INF-γ secretion. Chemokines such as IL-8, MCP-1, and MIP-1β, have also been shown to recruit neutrophils and lymphocytes to the site of mycobacterial infections. We have studied IL-8 expression in relation to TNF-α and TGF-β in monocytes from lepromatous patients (LL) as compared with healthy endemic controls. In endemic controls, no spontaneous expression of IL-8, TNF-α, and TGF-β was observed, but BCG and *M. leprae* induced activation of all three cytokines. Lepromatous leprosy monocytes spontaneously expressed high levels of IL-8 and TGF-β but negligible levels of TNF-α. A further increase in IL-8 secretion or gene expression by BCG or *M. leprae* was not significant. BCG, but not *M. leprae*, was able to stimulate TNF-α activation in lepromatous leprosy subjects. TGF-β responses in LL were parallel to those of IL-8. This suggests a vigorous and active ongoing IL-8 response in lepromatous disease that is independent of TNF-α activation. Therefore, in the absence of IFN-γ and TNF-α activation, IL-8 may assume a pivotal role in cell recruitment in leprosy patients with disseminated mycobacterial infections.

RÉSUMÉ

Le facteur de nécrose tumorale (TNF-α), associé à l'interféron-γ (INF-γ), joue un rôle important pour le recrutement des lymphocytes et la formation des granulômes dans les maladies à mycobactéries. Les infections de lèpre lépromateuse sont habituellement associées à des réponses faibles à nulles de lymphocytes T et une absence de sécrétion d'IFN-γ. Il a aussi été démontré que les chimiokines comme l'Il-8, la MCP-1 et le MIP-1β pouvaient permettre le recrutement de neutrophiles et de lymphocytes au sein des sites d'infection par les mycobactéries. Nous avons étudié ici l'expression de l'IL-8, en relation avec TNF-α et TGF-β, des monocytes de patients lépromateux (LL) et de témoins sains en zone endémique. Chez les témoins de zone endémique, il ne fut pas observé d'expression spontanée d'IL-8, de TNF-α ni de TNF-β, cependant tant le BCG que *M. leprae* ont provoqué l'activation de ces 3 cytokines. Les monocytes des patients souffrant de lèpre lépromateuse exprimaient spontanément de hauts niveaux d'IL-8 et de TGF-β mais une quantité négligeable de TNF-α. L'augmentation de sécrétion ainsi que la sur-expression du gène de l'IL-8 ne s'est pas avérée significative, après stimulation par le BCG ou *M. leprae*. Le BCG, mais pas *M. leprae*, a été capable de stimuler l'activation de TNF-α parmi les sujets atteints de lèpre lépromateuse. Les réponses de TGF-β chez les LL étaient très similaires à celles de l'IL-8. Cela suggère qu'il existe une médiation vigoureuse, active et continue de l'IL-8 dans la maladie lépromateuse, qui est indépendante de l'activation du TNF-α. L'IL-8 pourrait donc bien assurer un rôle central de recrutement cellulaire chez les patients atteints de lèpre présentant une infection massive et disséminée, en l'absence d'activation de l'IFN-γ et de TNF-α.

RESUMEN

El factor de necrosis tumoral alfa (TNF-α), junto con el interferón gamma (IFN-γ), juega un papel importante en el reclutamiento de linfocitos y en la formación de granulomas en las

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[2] Z. Hasan, Ph.D.; A. Mahmood, M.Sc., Department of Pathology and Microbiology, The Aga Khan University; S. Zafar, MBBS ; A. A. Khan, MBBS, Marie Adelaide Leprosy Center, Karachi, Pakistan; and R. Hussain, Ph. D., FRC(Path.), Department of Pathology and Microbiology, The Aga Khan University, Karachi, Pakistan.

Reprint requests: Zahra Hasan, Ph.D., Assistant Professor, Department of Pathology and Microbiology, The Aga Khan University, P.O. Box 3500, Karachi 74800, Pakistan. Email: zahra.hasan@aku.edu

enfermedades por micobacterias. La lepra lepromatosa está típicamente asociada con una baja o ausente respuesta inmune celular y con nula secreción de IFN-γ. Las quimiocinas tales como IL8, MCP-1 y MIP-1β, también reclutan neutrófilos y linfocitos en los sitios de infección. En esta investigación estudiamos la expresión de IL-8 en relación al TNF-α y al TGF-β en monocitos de pacientes con lepra lepromatosa (LL) y de controles sanos de áreas endémicas. En los controles endémicos no se observó la expresión espontánea de IL-8, TNF-α y TNF-β, pero BCG y *M. leprae* indujeron la activación de estas 3 citocinas. Los monocitos de los pacientes lepromatosos, en cambio, expresaron de manera espontánea, niveles elevados de IL-8 y TGF-β así como cantidades insignificantes de TNF-β. El estímulo con BCG o *M. leprae* no condujo a un incremento significativo en la secreción de IL-8. BCG, pero no *M. leprae*, fue capaz de estimular la activación de TNF-α en los pacientes con lepra. Las respuestas en TGF-β en los pacientes LL fueron equiparables a las respuestas en IL-8. Esto sugiere una vigorosa y activa respuesta en IL-8 en la lepra LL, que es independiente de la activación del TNF-α. Por lo tanto, en ausencia de la activación de IFN-γ y TNF-α, la IL-8 puede asumir un papel central en el reclutamiento de células en los pacientes con infecciones micobacterianas diseminadas.

Leprosy is a spectral disease with severity ranging from the localized or tuberculoid form to the disseminated or lepromatous form at the two poles, with several intermediate forms. The presentation of leprosy depends on the varying immune status of the infected individual ([27]). At the tuberculoid pole the disease is more restricted and bacilli tend to be contained in well-defined granulomas. At the lepromatous pole the disease is characterized by diffuse multibacillary lesions containing an extensive bacterial load in tissue macrophages especially, in the skin and also in Schwann cells of the peripheral nerves. Cytokine activation is critical to recruitment of a protective inflammatory response by macrophages and other polymorphonuclear leucocytes (PMNs) at the site of infection in the initial stages of the disease, for granuloma formation and limiting spread of infection. Monocytes-derived tumor necrosis factor alpha (TNF-α) ([16]) and T-cell derived interferon gamma (IFN-γ) play a pivotal role in the restriction of *Mycobacterium leprae* infections ([33]). In chronic disseminated lepromatous disease there is T cell anergy and low to absent IFN-γ responses as well as low levels of TNF-α secretion from peripheral blood mononuclear cells (PBMCs) in response to either mitogenic ([7]) or *M. leprae* components ([3]), as compared with the tuberculoid form of the disease.

In lepromatous patients, chemokines may assume a greater role in cell recruitment where there are low levels of T cell and monocyte derived pro-inflammatory cytokines. Leucocyte-attracting chemokines such as, the CXC chemokine interleukin-8 (IL-8), macrophage chemotactic protein-1 (MCP-1), and macrophage inhibitory protein-1β (MIP-1β), produced by macrophages, epithelial and mast cells have been shown to play a role in recruitment of neutrophils and lymphocytes in mycobacterial infections ([14, 19, 32]). IL-8 is stimulated in response to *M. tuberculosis* and its cellular components ([23, 31, 39, 41]), *M. bovis* ([24]) and *M. avium* ([38]). It has been shown to selectively recruit effector leucocyte populations to tuberculous granulomas ([5]). However, little is known about the role of chemokines in disseminated leprosy. In the current study we have focused on IL-8 responses in relation to TNF-α in both uninfected and infected (BCG and *M. leprae)* monocytes from lepromatous patients and compared them with healthy controls. In addition, we determined expression of the pleiotropic cytokine TGF-β which is thought to be immunosuppressive role in leprosy ([15]) and tuberculosis ([12]) infections. We found selective up-regulation of IL-8 but not of TNF-α in patients with lepromatous leprosy disease (LL), concomitant with increased TGF-β. Infection with BCG or *M. leprae* activated IL-8 responses but this was not significant due to the high basal level of IL-8. *Mycobacterium*-induced TNF-α activation was reduced as compared with endemic controls. These results suggest that IL-8 responses are active in these anergic patients and may contribute to disease pathology.

MATERIALS AND METHODS

Leprosy subjects and controls. Leprosy patients were recruited in collaboration

with the Marie Adelaide Leprosy Center, Karachi, Pakistan, and classified based on clinical presentation using World Health Organization (WHO) criteria ([30]). Fourteen subjects with lepromatous leprosy (LL), thirteen males and one female, were included in the study with informed consent. The age range of patients was 25 to 65 yrs with a mean of 34.4 yrs. Bacterial index (BI) of patients was assessed by slit skin smears. Patients were further divided into a short-term (ST) treatment group of MDT ≤1 month or, a long-term (LT) treatment group of multi-drug therapy (MDT) 2 to 6 months. Their clinical characteristics are further described in Table 1. BCG-vaccinated healthy volunteers (N = 14), 10 males and 4 females, ranging 25 to 50 yrs, with a mean age 32.4 yrs were included as a control group.

Mycobacterial strains. *M. bovis* BCG (Montreal vaccine strain) was kindly provided by Dr. Douglas Young, Imperial College, U.K. Mycobacteria were grown to logarithmic phase in 7H9 Middlebrook medium supplemented with 0.02% glycerol, 10% ADC Middlebrook enrichment and 0.5% Tween-80 (Difco Laboratories, Detroit, MI, U.S.A.). BCG were quantitated by growth on 7H10 Middlebrook agar, supplemented with 0.02% glycerol and 10% OADC Middlebrook enrichment (Difco Laboratories, Detroit, MI, U.S.A.). Mycobacteria were frozen in growth medium containing 20% glycerol as single use aliquots at –70°C. On recovery, the frozen stocks were found to be greater than 95% viable as determined by colony forming units (CFUs) and flouresceindiacetate-ethidium bromide staining ([18]). For the infection assays, aliquots of BCG were freshly thawed, washed 3 times in phosphate buffered saline (PBS) and diluted as required for the infection. To avoid clumping of mycobacteria, the cell suspension was sonicated briefly then allowed to stand for 5 min to allow large clumps to settle, leaving behind a mainly single cell suspension (as determined by acid-fast staining). Gamma-irradiated *M. leprae* (prepared from armadillo liver tissue) was provided by Dr. Patrick Brennan, Colorado State University, U.S.A., by National Institute of Health (NIH) contract (NO1-AI-55262, "Leprosy research support") as a 1 mg/ml stock containing approximately 2.9×10^9 mycobacteria/ml. The *M. leprae* was stored in single use aliquots at –70°C, and was washed in PBS, diluted as required as described above for the infection assays.

TABLE 1. *Characteristics of lepromatous leprosy (LL) patients included in study.*

No. of subjects (N)	Average age (yrs)	Mean BI	Mean MDT (mos.)
Short-term treatment group (LL-ST)			
7	29.9	2.75	0.28
Long-term treatment group (LL-LT)			
7	35	3.18	3.92

BI, bacterial index at time of enrollment in the study; MDT, duration of multi-drug treatment at time of enrollment.

Isolation and infection of monocytes. PBMCs were obtained by gradient separation of whole blood using Histopaque (Gibco BRL, NY, U.S.A.). Monocytes were isolated by adherence for 1.5 hr at 37°C after which cells were harvested using Cell Dissociation Solution (Sigma), counted and seeded at 5×10^5 per well in 24-well plates. Monocytes were cultured for 18 to 20 hours in RPMI 1640 medium containing heat-inactivated 10% human AB Serum (Sigma) and 2.5 mM glutamine. An optimal mycobacterium : monocyte ratio was first determined for the infection assays as described previously ([11]). Subsequent infection assays were carried out at a ratio of 10 bacteria per cell.

ELISA for TNF-α and IL-8. TNF-α standards and monoclonal antibody pairs for capture and detection were obtained from Pharmingen (San Diego, CA, U.S.A.). TNF-α was measured using a sandwich ELISA technique according to the manufacturer's recommendation and as reported previously ([13]). IL-8 detection reagents were obtained from R&D Systems, U.S.A., and were also used in a sandwich ELISA method according to manufacturer's recommendation. The range of detection was between 7.8 to 1000 pg/ml. Each sample was tested in duplicate. Results are expressed as mean values from at least four independent experiments after deduction of spontaneous cytokine production. As expected, donor to donor variation was present but results were consistent and were pooled for data analyses.

Statistical analyses were carried out using the Microsoft EXCEL Program. Values in each group of results were compared using ANOVA analysis. Un-paired Student's *t*-test was carried out with 95% confidence intervals so that p-values ≤0.05 were considered significantly different.

RT-PCR for cytokines. Cell monolayers were directly harvested in TriZOL reagent (GIBCO BRL) and total RNA, isolated according to the manufacturer's recommendations. Reverse transcription of 1 to 2 μg RNA was carried out using oligo dT primers at 37°C for 2 hrs with Murine Leukemia Virus transcriptase (GIBCO-BRL). Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out using sequence specific primers for the house keeping β-actin gene, IL-8, TNF-α, and TGF-β. PCR was first carried out for β-actin and subsequently, equivalent cDNA template was used in the PCR reactions. Primers used were: β-actin-F 5′ GTG GGG CGC CCC AGG CAC CA 3′, -R 5′ CTC CTT AAT GTC ACG CAC GAT TTC 3′); TNF-αF (5′ TCT CGA ACC CCG AGT GAC AA 3′), -R (5′ TAT CTC TCA GCT CCA CGC CA 3′); TGF-β-F (5′ GCC CTG GAC ACC AAC TAT TGC 3′), -R (5′ GCT GCA CTT GCA GGC GCG CAC 3′) and commercially available primers for IL-8 (R&D systems, U.S.A.). PCR conditions for IL-8 and TNF-α were briefly: 94°C, 2 min; 30 cycles, 94°C, 45 seconds; 56°C, 45 seconds; 72 seconds, 1 min; 72°C, 10 min. Annealing temperatures for β-actin and TGF-β were 58°C. The mRNA products of sizes 540 bp (β-actin), 158 bp (IL-8), 124 bp (TNF-α) and 336 bp (TGF-β) were detected. Gene expression was normalized using β-actin, whereby the PCR products obtained were quantitated by scanning densitometry scanned using the BIORAD Gel Documentation system (BIORAD, U.S.A.), which provides arbitrary units for the relative intensity of each band obtained. Densitometry results were obtained for each test sample for cytokine PCRs and gene expression indicated as cytokine/β-actin ratios.

RESULTS

Differential induction of IL-8 and TNF-α secretion in lepromatous patients. We first determined basal levels of IL-8 and TNF-α expression in our group of lepromatous leprosy patients (LL) and in endemic controls. Very little spontaneous secretion of IL-8 was observed in healthy donors, while that in lepromatous patients was significantly elevated ($p < 0.05$) (Fig. 1A). However, there was no spontaneous secretion of TNF-α observed in either healthy donors or patients (Fig. 1B). Since chemotherapy may change the immune profile, we also analyzed the effects of duration of treatment on cytokine secretion. There was no significant difference in IL-8 secretion in short term treated (ST) or long-term (LT) treated LL patients (Fig. 1A). There was a higher trend of TNF-α secretion in LL-LT patients as compared with the LL-ST group (Fig. 1B), which may be related to therapy but because of the donor to donor variation and small group size this did not achieve statistical significance ($p = 0.49$). Therefore, it appeared that there was a vigorous IL-8 response in LL patients, which was not related to a generalized activation of the monocytes.

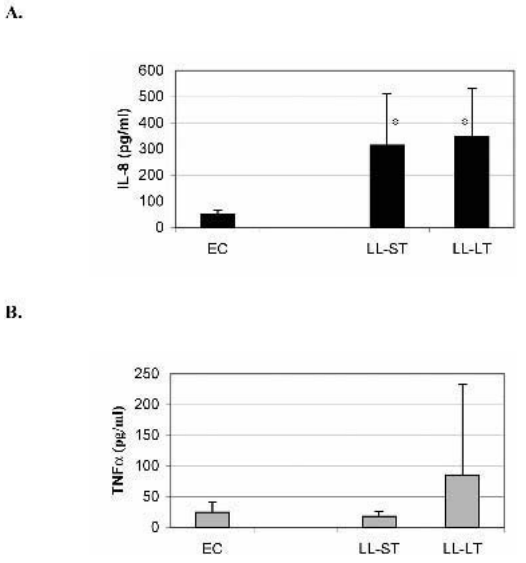

FIG. 1. IL-8 but not TNF-α is spontaneously secreted in LL patients. Monocytes were cultured for 18–24 hr after which the medium was replenished. IL-8 and TNF-α activity was measured in supernatants collected after 6 hr. Data is shown for EC (N = 7); short-term treated leprosy patients (LL-LT), N = 4 and long-term treated leprosy patients (LL-ST), N= 6. The mean values are illustrated with S.E.M. as 'y' error. A. IL-8, B. TNF-α. Significance of differences between LL groups and ECs were determined using an unpaired Student's *t*-test. *, $p \leq 0.05$ as significant.

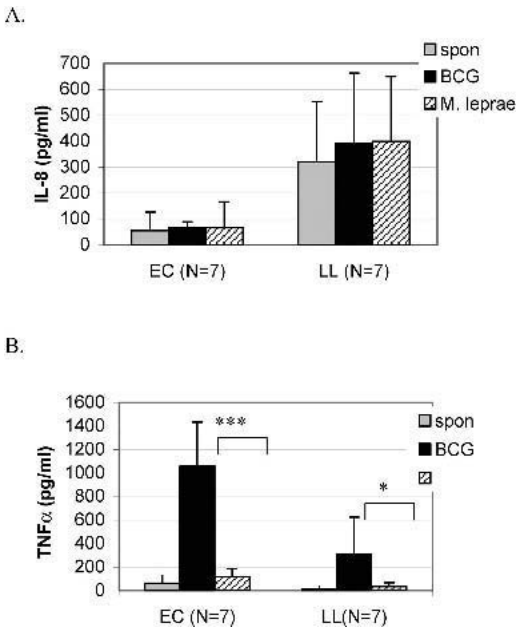

FIG. 2. IL-8 and TNF-α are differentially induced in BCG and *M. leprae* stimulated monocytes from LL patients. Monocytes (5×10^5) were infected with BCG at a ratio of 10 per cell. Cells supernatants were harvested at 6 hr post-infection and measured for IL-8 and TNF-α activity. Spontaneous, BCG-, and *M. leprae*-induced activation is shown for EC and LL subjects. Mean values are shown with S.E.M. as 'y' error. *, p ≤0.05; ***, p ≤0.001. A. IL-8, B. TNF-α.

To determine whether cytokines was further inducible in monocytes from leprosy patients, we infected cells with live, attenuated *M. bovis* BCG or irradiated *M. leprae*. Previously, we have found 6 hr post-infection to be an optimal time to measure mycobacterium-induced TNF-α ([11]). For IL-8 we carried out a time course infection of monocytes at 1, 6, and 18 hr, and found negligible IL-8 at 1 hr with a subsequent increase in IL-8 secretion up to 18 hr post stimulation (data not shown). Therefore, we used the 6 hr time point to detect IL-8 in our system in between endemic controls and LL subjects. In addition, this allowed us to compare IL-8 and TNF-α in the same interval.

As we had observed a trend of higher spontaneous secretion of TNF-α in LL-LT group (Fig. 1), we also compared mycobacterium-induced TNF-α and IL-8 activation between the LL-ST and LL-LT groups (data not shown). Neither TNF-α (p = 0.22) nor IL-8 (p = 0.87) secretion between LL-ST and LL-LT groups was found to be significantly different using Students *t*-test analysis, suggesting that both treatment groups showed similar responses and for the purpose of this study could be studied together.

Both BCG and *M. leprae* were able to induce incremental responses in LL patients over the ongoing IL-8 response (Fig. 2A). There was little increase in IL-8 secretion observed in endemic controls in response to mycobacterium stimulation. The overall IL-8 responses in LL patients were 3 to 5 fivefold higher than in endemic controls. In contrast, monocytes from both endemic controls and lepromatous patients showed significantly greater TNF-α production in response to BCG stimulation as compared with *M. leprae* (Fig. 2B). With, *M. leprae*-induced TNF-α found to be negligible in the LL group. In comparison to the endemic controls and LL groups, BCG-induced TNF-α activation was significantly greater in endemic controls (p = 0.001) than in LL. These results clearly demonstrate that there is an overall increase in IL-8 secretion in LL patients which is independent of TNF-α responses.

BCG and *M. leprae* induced gene expression in endemic contols and LL subjects. We next determined cytokine gene expression induced by BCG and *M. leprae*. In addition to IL-8 and TNF-α, we also monitored TGF-β expression, which has previously been shown to be raised in lepromatous infections ([10, 15]). We first looked at early gene expression at 1 hr post-infection in endemic controls. Spontaneous gene expression of TNF-α and IL-8 was negligible in endemic controls (Fig. 3), corresponding with protein data described (Fig. 1). BCG induced slightly greater IL-8 activation than *M. leprae*, while TNF-α mRNA expression in response to BCG and *M. leprae* was comparable at 1 hr. This was in contrast to the greater TNF-α secretion induced by BCG (Fig. 2). There was some TGF-β mRNA activation present in unstimulated which was transcriptionally upregulated similarly by both BCG and *M. leprae* stimulation.

We subsequently determined a time course of cytokine gene expression profiles in leprosy patients. Spontaneous IL-8 gene expression was detectable within 1 hr and was maintained up to 18 hr, while BCG and

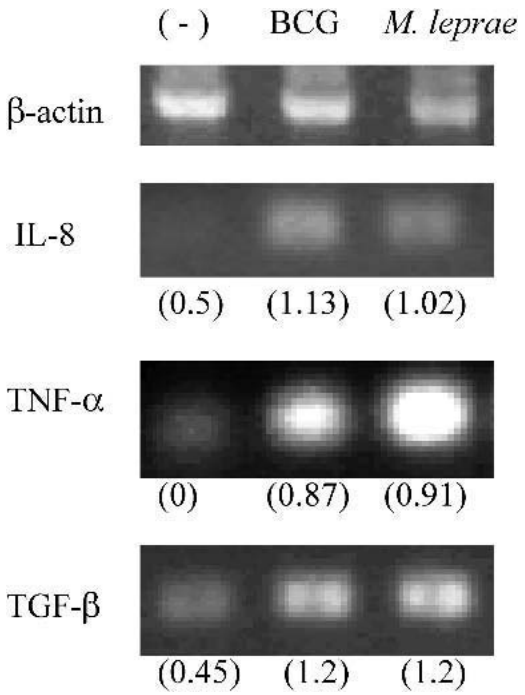

FIG. 3. BCG- and *M. leprae*-induced cytokine gene expression in ECs. Monocytes were infected with BCG and *M. leprae* and total cellular RNA was extracted at 1 hr post-infection, subjected to RT-PCR using sequence specific primers. Panels show gene expression in the absence of stimulus (–) and after infection with BCG or *M. leprae*. Gene expression was quantitated by normalizing with β-actin and the cytokine expression/β-actin ratios are given in parentheses. Profiles of β-actin, IL-8, TNF-α and TGF-β of one EC subject are shown. This was representative of 3 independent experiments.

M. leprae induced IL-8 activation at all time points studied (Fig. 4). Spontaneous activation of TNF-α was negligible and TNF-α mRNA expression was only induced by BCG at 6 hr post-infection, while no *M. leprae*-induced stimulation was observed. This was in contrast to the early gene activation seen in monocytes from endemic controls (Fig. 3). TGF-β was spontaneously activated as early as 1 hr and remained expressed until 18 hr post-infection, both in the presence and absence of BCG and *M. leprae* infection in leprosy patients. The overall frequency of gene expression in both endemic controls and LL groups is given in Table 2. These results clearly demonstrate an overall heightened activation of IL-8 and TGF-β in LL patients which is not linked to TNF-α.

DISCUSSION

Lepromatous leprosy infection is characterized by the absence of well-defined granuloma formation at the site of infection. Although both macrophages and neutrophils are found at the site of infection, critical cytokines required for granuloma formation (TNF-α and IFN-γ) are absent in these patients ([2]). Leucocyte chemoattractants such as, IL-8, MCP-1, and MIP-1 are involved in cell recruitment in the early phase of infection and there is evidence that they may be involved in the chronic phase of mycobacterial disease ([8, 21]). In the current study we show spontaneous mRNA expression of IL-8 and TGF-β from monocytes of LL patients in the absence of TNF-α, suggesting an ongoing *in vivo* response.

Although our LL patients had been variably treated (0 to 6 months) the mean BI of the LL-ST and LL-LT groups was similar. In addition, the limited chemotherapy used had little effect on ongoing spontaneous IL-8 and TNF-α response, or on the BCG or *M. leprae*-induced responses as compared between the groups (data not shown). These results led us to consider the LL patients as a single group for this current analysis.

Spontaneous IL-8 in monocytes from lepromatous patients patients has not been previously reported, although IL-8 mRNA has been observed mainly in patients undergoing reactions ([26]) where it is thought that it may have a role in generating the pathology associated with leprosy reactions. Reduced TNF-α correlates with previous reports of low proinflammatory cytokines found in LL subjects ([40]). Due to the high basal IL-8 levels in LL patients, it was not surprising to observe little incremental increase in BCG and *M. leprae* induced IL-8 mRNA expression and secretion. This phenomenon of lowered IL-8 inducibility has been previously described by Friedland, *et al*. in leucocytes of patients who suffered from fatal tuberculosis and were unable to respond to lipo-polysaccharide (LPS) stimulation by appropriate secretion of TNF-α or IL-8, despite having raised serum levels of IL-8 ([8]).

In comparison of BCG- and *M. leprae*-cytokine activation, the latter consistently induced lower IL-8 and TNF-α secretion in both LL and endemic controls groups. The

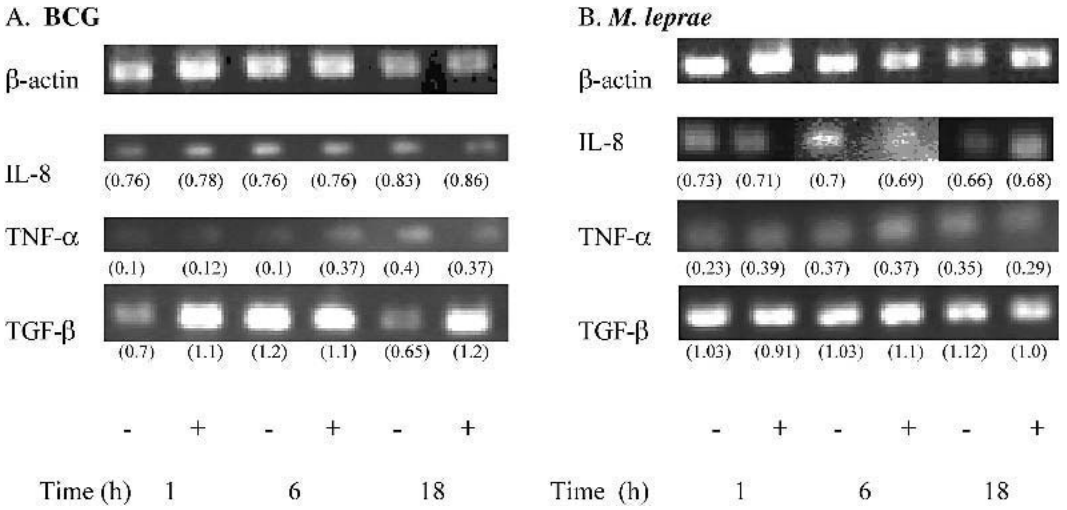

FIG. 4. Time course of BCG and *M. leprae*-induced cytokine gene expression in leprosy. Monocytes were infected with BCG and *M. leprae* for 1, 6 and 18 hr as described in Fig. 3. Gene expression profiles for one representative leprosy patient each in the absence (–) and presence (+) of mycobacterial stimulation are indicated with cytokine/β-actin expression ratios. A. BCG, B. *M. leprae.*

IL-8 levels we observed in EC are lower than those reported by Mendez-Samperio, *et al*. ([25]). Differences in cytokine production can be attributed to mycobacterial strain variations, as we used the BCG Montreal vaccine strain while, Mendez-Samperio, *et al*. employed the BCG Danish vaccine strain. In addition, immune responses of healthy subjects from mycobacterial endemic regions vary from those of non-endemic regions. In endemic controls, we observed TNF-α secretion in response to BCG but none to *M. leprae,* correlating with previous reports that *M. leprae* has been shown to be a poor stimulator of immune activating cytokines IL-1, TNF-α and IL-6 ([28, 37]). This cannot be attributed to a difference in viability of the two species, as although the *M. leprae* was γ-irradiated, it has been shown to be a reliable tool as this method of killing is thought to preserve cell wall structure and therefore, antigenicity of the bacillus ([1]). In addition, previous studies have also shown that *M. leprae* live and dead induced similar cytokine responses ([37]).

However, we found *M. leprae*–induced TNF-α mRNA at 1 hr post-infection *M. leprae* in endemic controls, which correlates with reports by Shimizu, *et al*. ([34]). This discrepancy may possibly be due to post-transcriptional down-regulation of TNF-α by *M. leprae.* As, *M. leprae* triggers host cellular signaling pathways tyrosine kinases ([22]) and mitogen activated protein kinase pathways ([11]) upon attachment and uptake into cells, which can in turn downregulate TNF-α activation.

The absence of TNF-α in LL at the disease site ([2]) despite high bacterial load, and in PBMCs from LL patients in response to *M. leprae* ([3]), is therefore not surprising. However, the high spontaneous secretion of IL-8 in LL patients is suggests that other factors *in vivo* may be modulating these monocytes. We observed spontaneous IL-8 gene expression in all of the LL patients studied, which is consistent with the high levels of IL-8 protein secretion detected. A time course gene expression profile in LL patients showed that the relatively low TNF-α stimulation in response to both

TABLE 2. *Gene expression of IL-8, TNF-α and TGF-β in LL subjects.*

	IL-8*		TNF-α*		TGF-β*	
	EC	LL	EC	LL	EC	LL
Spon.	0 (4)	5 (6)	0 (4)	3 (4)	0 (3)	4 (4)
BCG	2 (2)	4 (4)	2 (2)	3 (3)	2 (2)	3 (3)
M. leprae	3 (3)	4 (4)	1 (2)	2 (3)	2 (2)	3 (3)

* Positive samples expressed distinct relevant bands in the PCR reactions : Number of samples positive for gene expression (total number of samples).

BCG and *M. leprae* was not due to suboptimal time of detection in the assay.

Our data indicates that mycobacterium-induced IL-8 activation in leprosy is independent of TNF-α. Previous studies show that TNF-α can enhance IL-8 activity ([29, 36]), but activation of IL-8 and TNF-α can also occur via different pathways, dependent on the stimulus ([6]). Our results correlate with reports that *M. tuberculosis*-induced IL-8 secretion is independent of TNF-α ([9]).

It is the coordinated activation of cytokines that is responsible for the immune profile present in response to mycobacterial infections. TGF-β, is a pleiotropic cytokine important in macrophage immunomodulation and involved in tissue healing and repair ([20]). Raised TGF-β in leprosy disease especially, in lepromatous infections is thought to be associated with a suppressive action locally ([15, 17]). TGF-β can inhibit IL-1 and TNF-α secretion in macrophages depending on the stimulus ([4]). Therefore, the raised constitutive levels of TGF-β we find in the LL subjects may be responsible for the low TNF-α expression observed. In addition, while TGF-β has been shown to downregulate TNF-α induced IL-8 production in endothelial cells ([35]), it is unable to inhibit *Mycobacterium*-induced IL-8 production ([24]). Which, may explain the raised IL-8 response despite endogenous TGF-β activation.

Overall, our work shows that while other proinflammatory cytokines may be reduced in the anergic lepromatous patient, the host innate response involving the leucocyte attractant IL-8 is still vigorously ongoing. This may contribute to cell recruitment in severe forms of leprosy even though it may be inappropriate and ineffective and may instead result in pathology rather than protection in the more chronic phase of leprosy infections.

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REFERENCES

1. Adams, L. B., Soileau, N. A., Battista, J. R., and Krahenbuhl, J. L. Inhibition of metabolism and growth of *Mycobacterium leprae* by gamma irradiation. Int. J. Lepr. Mycobact. Dis. **68** (2000) 1–10.
2. Arnoldi, J., Gerdes, J., and Flad, H. D. Immunohistologic assessment of cytokine production of infiltrating cells in various forms of leprosy. Am. J. Path. **137** (1990) 749–753.
3. Barnes, P. J., Chatterjee, D., Brennan, P. J., Rea, T. H., and Modlin, R. L. Tumor necrosis factor production in patients with leprosy. Infect. Immun. **60** (1992) 1441–1446.
4. Chantry, D., Turner, M., Abney, E., and Feldmann, M. Modulation of cytokine production by transforming growth factor-β. J. Immunol. **142** (1989) 4295–4300.
5. Ferrero, E., Biswas, P., Vettoretto, K., Ferrarini, M., Uguccioni, M., Piali, L., Leone, B. E., Moser, B., and Rugarli, C. Macrophages exposed to *Mycobacterium tuberculosis* release chemokines able to recruit selected leucocyte subpopulations: focus on γδ cells. Immunol. **108** (2003) 365–374.
6. Foreback, J. L., Sarma, V., Yeager, N. R., Younkin, E. M., Remick, D. G., and Ward, P. A. Blood mononuclear cell production of TNF-α and IL-8: engagement of different signal transduction pathways including the p42 MAP kinase pathway. J. Leukoc. Biol. **64** (1998) 124–133.
7. Foss, N. T., de Oliveria, E. B., and Silva, C. L. Correlation between TNF production, increase of plasma C-reactive protein level and suppression of T lymphocyte response to concanavalin A during erythema nodosum leprosum. Int. J. Lep. **61** (1993) 218–226.
8. Friedland, J. S., Hartley, J. C., Hartley, C. G., Shattock, R. J., and Griffin, G. E. Inhibition of *ex vivo* proinflammatory cytokine secretion in fatal *Mycobacterium tuberculosis* infection. Clin. Exp. Immunol. **100** (1995) 233–238.
9. Friedland, J. S., Remick, D. G., Shattock, R. J., and Griffin, E. Secretion of interleukin-8 following phagocytosis of *Mycobacterium tuberculosis* by human monocyte cell lines. Eur. J. Immunol. **22** (1992) 1373–1378.
10. Goulart, I. M. B., Mineo, J. R., and Foss, N. T. Production of transforming growth factor-beta 1 (TGF-β1) by blood monocytes from patients with different clinical forms of leprosy. Clin. Exp. Immunol. **122** (2000) 330–334.
11. Hasan, Z., Shah, B. H., Mahmood, A., Young, D. B., and Hussain, R. The effect of mycobacterial virulence and viability on MAP kinase signaling and TNF-α production by human monocytes. Tuberculosis **83** (2003) 299–309.
12. Hirsch, C. S., Hussain, R., Toosi, Z., Dawood, G., Shahid, F., and Ellner, J. J. Cross modulation by transforming growth factor β in human tuberuclosis: suppression of antigen-driven blatogenesis and interferon-γ. Proc. Natl. Acad. Sci. USA **94** (1996) 3926–3931.
13. Hussain, R., Shiratsuchi, H., Philips, M., Ell-

NER, J., and WALLIS, R. S. Opsonizing antibodies (IgG1) up-regulate monocyte proinflammatorycytokines tumour necrosis factor-alpha (TNF-α) and IL-6 but not anti-inflammatory cytokine IL-10 in mycobacterial antigen-stimulated monocytes—implications for pathogenesis. Clin. Exp. Immunol. **123** (2001) 210–218.

14. KASAHARA, K., SATO, I., OGURA, K., TAKEUCHI, H., KOBAYASHI, K., and ADACHI, M. Expression of chemokines and induction of rapid cell death in human blood neutrophils by *Mycobacterium tuberculosis*. J. Infect. Dis. **178** (1998) 127–137.
15. KHANOLKAR-YOUNG, S., SNOWDON, D., and LOCKWOOD, D. N. J. Immunocytochemical localization of inducible nitric oxide synthease and transforming growth factor-beta (TGF-β) in leprosy lesions. Clin. Exp. Immunol. **113** (1998) 438–442.
16. KINDLER, V., SAPPINO, A. P., GRAU, G. E., PIGUET, P. F., VIA, L. E., and VASSALLI, P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell **56** (1989) 731–740.
17. KISZEWSKI, C. A., BECERRIL, E., BAQUERA, J., AUGUILAR, L. D., and HERNANDEZ-PANDO, R. Expression of transforming growth factor-β isoforms and their receptors in leprmatous and tuberculoid leprosy. Scand. J. Immunol. **57** (2003) 279–285.
18. KVACH, J. T., and VERAS, J. R. A fluorescent staining procedure for determining the viability of mycobacterial cells. Int. J. Lep. **50** (1982) 183–192.
19. LARSEN, C. G., ANDERSON, A. O., APPELLA, E., OPPENHEIM, J. J., and MATSUSHIMA, K. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. Science **243** (1989) 1464–1466.
20. LETTERIO, J. J., and ROBERTS, A. B. Regulation of immune responses by TGF-β. Annu. Rev. Immunol. **16** (1998) 137–161.
21. LEW, W., CHANG, S., TADA, Y., NAKMURA, K., and TAMAKI, K. Serum monocyte chemoattractant protein-1 is elevated in lepromatous leprosy patients with high bacterial indices. Int. J. Lep. **70** (2002) 129–131.
22. LIMA, C. S., RIBEIRO, M. L., SOUZA, L. A., SARDELLA, A. B., WOLF, V. M. A., and PESSOLANI, M. C. V. Intracellular signals triggered during association of *Mycobacterium leprae* and *Mycobacterium bovis* BCG with human monocytes. Microbiol Pathogen. **31** (2001) 37–45.
23. LIN, Y., ZHANG, M., and BARNES, P. F. Chemokine production by a human alveolar epithelial cell line in response to *Mycobacterium tuberculosis*. Infect. Immun. **66** (1998) 1121–1126.
24. MENDEZ-SAMPERIO, P., GARCIA, E., VAZQUEZ, A., and PALMA, J. Regulation of interleukin-8 by interleukin-10 and transforming growth factor β in human monocytes infected with *Mycobacterium bovis*. Clin. Diag. Lab. Immunol. **9** (2002) 802–807.
25. MENDEZ-SAMPERIO, P., PALMA, J., and VAZQUEZ, A. Roles of intracellular calcium and NF-kappaB in the Bacillus Calmette-Guerin—induced secretion of interleukin-8 from human monocytes. Cell. Immunol. **211** (2001) 113–122.
26. MORAES, M. O., SARNO, E. N., ALMEIDA, A. S., SARAIVA, B. C. C., NERY, J. A. C., MARTINS, R. C. L., and SAMPAIO, E. P. Cytokine mRNA expression in leprosy: a possible role for interferon-γ and interleukin-12 in reactions (RR and ENL). Scand. J. Immunol. **50** (1999) 541–549.
27. MYRVANG, B., GODAL, T., RIDLEY, D. S., FROLAND, S. S., and SONG, Y. K. Immune responsiveness to *Mycobacterium leprae* and other mycobacterial antigens throughout the clinical and histopathological spectrum of leprosy. Clin. Exp. Immunol. **14** (1973) 541–553.
28. NAKAMURA, C., FUKUTOMI, Y., KASHIWABARA, Y., OOMOTO, Y., KOJIMA, M., HAYASHI, H., and ONOZAKI, K. Dharmendra antigen but not integral *M. leprae* is an efficient inducer of immunostimulant cytokine production by human monocytes, and *M. leprae* lipids inhibit the cytokine production. Int. J. Lepr. Mycobact. Dis. **65** (1997) 63–72.
29. OSAWA, Y., NAGAKI, M., BANNO, Y., BRENNER, D. A., ASANO, T., NOZAWA, Y., MORIWAKI, H., and NAKASHIMA, S. Tumor necrosis factor alpha-induced interleukin-8 production via NF-kB and phosphatidylinositol 3-kinase/Akt pathways inhibits cell apoptosis in human hepatocytes. Infect. Immun. **70** (2002) 6294–6301.
30. RIDLEY, D. S., and JOPLING, W. H. Classification of leprosy according to immunity. Int. J. Lep. **34** (1966) 255–273.
31. RIEDEL, D. D., and KAUFMANN, S. H. E. Chemokine secretion by human polymorphonuclear granulocytes after stimulation with *Mycobacterium tuberculosis* and lipoarabinomannan. Infect. Immun. **65** (1997) 4620–4623.
32. SADEK, I., SADA, E., TOOSI, Z., SCHWANDER, S. K., and RICH, E. A. Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. Am. J. Respir. Crit. Care Med. **19** (1998) 513–521.
33. SASIAIN, M. D., DE LA BARRERA, S., VALDEZ, R., and BALINE, L. M. Reduced suppressor cell response to *Mycobacterium leprae* in lepromatous leprosy. Infect. Immun. **57** (1989) 951–956.
34. SHIMIZU, T., TOMIOKA, H., MATSUOKA, M., and SANO, C. Profiles of the mRNA expression by macrophages infected with *Mycobacterium leprae* and *Mycobacterium avium* complex. Int. J. Lep. **70** (2002) 250–259.
35. SMITH, W. B., NOACK, L., KHEW-GOODALL, Y., ISENMANN, S., VADAS, M. A., and GAMBLE, J. R. Transforming growth factor-β1 inhibits the production of IL-8 and the transmigration of neutrophils through activated endothelium. J. Immunol. **157** (1996) 360–368.
36. STRIETER, R. M., CHENSUE, S. W., BASHA, M. A., STANDIFOR, T. J., LYNCH, J. P., BAGGIOLINI, M., and

KUNKEL, S. L. Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor-alpha, lipopolysaccharide, and interleukin-1 beta. Am. J. Respir. Crit. Care Med. **2** (1990) 321–326.

37. SUZUKI, K., FUKUTOMI, Y., MATSUOKA, M., TORII, K., HAYASHI, H., TAKII, T., OOMOTO, Y., and ONOZAKI, K. Differential production of interleukin 1(IL-1), IL-6, tumor necrosis factor, and IL-1 receptor antagonist by human monocytes stimulated with *Mycobacterium leprae* and *M. bovis* BCG. Int. J. Lep. **61** (1993) 609–618.
38. WEISS, D. J., EVANSON, O. A., MORITZ, A., DENG, M. Q., and ABRAHAMSEN, M. S. Differential responses of bovine macrophages to *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. Infect. Immun. **70** (2002) 5556–5561.
39. WILKINSON, P. C., and NEWMAN, I. Identification of IL-8 as locomotor attractant for activated human lymphocytes in mononuclear cell cultures with anti-CD3 or purified protein derivative of *Mycobacterium tuberculosis*. J. Immunol. **149** (1992) 2689–2694.
40. YAMAMURA, M., UYEMUSA, K., DEANS, R., WEINBERG, K., REA, T., and BLOOM, B. R. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science **254** (1991) 277–279.
41. ZHANG, Y., BROSER, M., COHEN, H., BODKIN, M., LAW, K., REIBMAN, J., and ROM, W. N. Enhanced interleukin-8 release and gene expression in macrophages after exposure to *Mycobacterium tuberculosis* and its components. J. Clin. Invest. **95** (1995) 586–592.