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Erythema Nodosum Leprosum Neutrophil Subset Expressing IL-10R1 Transmigrates into Skin Lesions and Responds to IL-10

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ABSTRACT

Erythema nodosum leprosum (ENL) is an inflammatory complication in leprosy. Yet, the involvement of ENL neutrophils in the inflammatory response against *Mycobacterium leprae* remains poorly explored. Our primary aim was to investigate the utility of the surface expression of neutrophil IL-10R1 as an ENL biomarker and, secondarily, to evaluate whether leprosy or healthy *M. leprae*-stimulated neutrophils produce cytokines and are able to respond to IL-10. We, in this study, describe a subpopulation of circulating neutrophils of ENL patients that exclusively expressed IL-10R1, providing evidence that IL-10R1⁺ neutrophils are present in ENL lesions. It was also found that ENL neutrophils, but not those of nonreactive leprosy controls, were able to secrete detectable levels of TNF *ex vivo* and the addition of IL-10 blocked TNF release. It was likewise observed that *M. leprae*-stimulated, healthy neutrophils expressed IL-10R1 *in vitro*, and ENL-linked cytokines were released by *M. leprae*-cultured neutrophils *in vitro*. Moreover, consistent with the presence of a fully functional IL-10R, the addition of IL-10 prevented the release of *M. leprae*-induced cytokines. Most importantly, dead *M. leprae* revealed its superior capacity to induce *CCL4* and *IL-8* in primary neutrophils over live *Mycobacterium*, suggesting that *M. leprae* may hamper the inflammatory machinery as an immune escape mechanism. *ImmunoHorizons*, 2020, 4: 47–56.

INTRODUCTION

Leprosy is a chronic disease caused by *Mycobacterium leprae*, an obligate intracellular bacillus affecting the skin and peripheral nerves. Patients develop leprosy within a clinical spectrum ranging from tuberculoid leprosy (TT) through borderlines (borderline tuberculoid, borderline borderline, and borderline lepromatous [BL]) to lepromatous leprosy (LL) that are classified as such in

accordance with certain clinical, bacteriological, histopathological, and immunological criteria. TT patients have a specific cellular response against *M. leprae* in the form of a well-defined lesion with the presence of little or no bacillus. In contrast, LL patients present a robust and ineffective humoral response against the bacillus and a low or nonexistent cellular immune response, leading to intense mycobacterial proliferation resulting in disseminated lesions (1).

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Abbreviations used in this article: BL, borderline lepromatous; ENL, erythema nodosum leprosum; LL, lepromatous leprosy; MDT, multidrug therapy; MFI, median fluorescence intensity; RR, reversal reaction; TT, tuberculoid leprosy.

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During the chronic course of leprosy, patients may be afflicted with acute reactional episodes, thereby exacerbating their clinical condition by amplifying permanent nerve damage and physical disabilities. However, early therapeutic interventions are able to prevent the development of the disabilities associated with neuritis. Depending on the type of etiopathogenesis exhibited, these reactions are referred to as reversal reaction (RR) or erythema nodosum leprosum (ENL). But, whereas the cause(s) and mechanism(s) of both reactions remain elusive and ill defined, there is speculation that they could be associated with exacerbated cellular immunity, possibly triggered by a reaction to fragmented bacillary Ags (2).

ENL is characterized by the sudden appearance of inflammatory erythematous nodules located in the apparently normal skin and/or s.c. tissue involving a number of organs often associated with systemic manifestations. ENL lesions present a neutrophilic infiltrate accompanied by a cluster of foamy macrophages with a high bacillary load. Previous studies have shown that, during ENL, levels of such proinflammatory cytokines as TNF, IL-1 β , IL-6, and IFN- γ are elevated in patient circulation (3).

The immunomodulatory drug thalidomide is the treatment of choice for ENL. However, not all ENL patients have access to the drug. The administration of thalidomide leads to an early and significant improvement in clinical symptoms as well as a reduction in systemic proinflammatory molecules (4–6) and neutrophilic infiltration in the lesions (7). The therapeutic effect of thalidomide on ENL appears to be associated with transient immune stimulation, suggesting that the drug may promote an active immunoregulatory response (5, 8).

The IL-10 cytokine is widely known for its ability to attenuate inflammatory and immunological responses both *in vitro* and *in vivo*. It has already been shown that some functional activities of neutrophils are regulated by IL-10, as in LPS-induced cytokine production (9). IL-10 biological activity occurs when dimeric IL-10 binds to the heterodimeric receptor consisting of IL-10R1 and IL-10R2. Steady-state neutrophils do not respond to IL-10 in the absence of IL-10R1. This homeostatic IL-10 control mechanism arises under conditions of systemic inflammation (e.g., an LPS injection or sepsis), rendering the circulating neutrophils responsive to IL-10 because of the full expression of both the IL-10R1 and IL-10R2 chains (10). In this context, ENL neutrophils could serve as targets of IL-10 regulatory action. In view of the potential role of neutrophils in amplifying systemic inflammation during ENL, the ability of IL-10 to act as a potent modulator of this cell type opens new perspectives as to its potential therapeutic utility. Our hypothesis is that circulating neutrophils of ENL patients are responsive to IL-10 and express the IL-10R1 chain because of exposure to *M. leprae* and the inflammatory microenvironment.

MATERIALS AND METHODS

Ethics

Our study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines. Blood and

skin lesion samples were collected with prior approval from the Institutional Review Board of the Oswaldo Cruz Institute (CAAE 24006713.7.0000.5248 and 56113716.5.0000.5248). Sample collection was initiated after all the individuals and parents of underaged participants had been duly informed in regard to the study and rendered their consent.

Patients

The present case-control study was conducted at the Souza Araújo Outpatient Clinic, a reference center for leprosy diagnosis and treatment (Leprosy Laboratory, FIOCRUZ, Rio de Janeiro, RJ, Brazil). Leprosy was diagnosed via hypopigmented, anesthetic skin patches and/or thickened nerves and/or acid-fast bacilli on the slit-skin smears. Leprosy patients were classified according to the Ridley and Jopling Scale (1). ENL patients were defined as the ones diagnosed with leprosy that had an acute appearance of crops of tender cutaneous or s.c. lesions accompanied or not by fever, malaise, or any other symptomatic symptoms. These patients were treated with thalidomide (100–300 mg daily) in compliance with Brazilian Ministry of Health guidelines. Leprosy was treated with multidrug therapy (MDT) as recommended by the World Health Organization. The baseline characteristics of each group of patients enrolled in the study are shown in Table I.

Cell purification

Blood from the healthy donors and leprosy patients was collected in sterile collection tubes containing sodium heparin as an anticoagulant. Neutrophils were isolated by density gradient centrifugation at $800 \times g$ for 25 min on Ficoll-Paque (GE Healthcare Life Sciences), as described earlier (11). Remaining erythrocytes were lysed in isotonic ice-cold NH₄Cl solution, followed by centrifugation at 4°C. Cell viability was determined by trypan blue dye exclusion. Neutrophilic purity during final cell preparations was assessed by light microscopy of the cytocentrifuged preparations. Highly purified neutrophils were used for flow cytometry analyses and/or cell cultures.

Flow cytometry analysis

Neutrophils (1×10^6 total cells) were fixed with 4% paraformaldehyde, transported to a flow cytometry laboratory (Cytometry Platform, Oswaldo Cruz Institute, Rio de Janeiro, RJ, Brazil) during working hours or stored (4°C) in a refrigerated container. To evaluate the degree of purity, cells were stained with fluorescent CD16 Ab (clone eBioCB16; eBioscience). To evaluate IL-10R expression, cells were stained with unlabeled mouse anti-IL-10R1 (clone 37607.11; Santa Cruz Biotechnology) or rabbit anti-IL-10R2 Abs (clone G19; Santa Cruz Biotechnology), followed by incubation with a respectively specific secondary Ab, namely FITC anti-mouse or anti-rabbit IgG secondary Abs (DAKO). All Abs were titrated prior to experimental use. Cells were stained in the presence of human Fc receptor blocking. All flow cytometric analyses were carried out on FACSCalibur or BD Accuri C6 Flow Cytometer instruments (both from Becton Dickinson). A total of 10,000 events were collected for each sample. Data were processed by way of FlowJo software (Tree Star).

TABLE I. Demographic and clinical patient data

Characteristic	BL/LL ^a	ENL ^b
Individuals	28	28
Sex		
Male	20	21
Female	8	7
Age, y, median (min–max)	48.5 (22–64)	40.0 (20–76)
BI (median)	4.55	3.6

^aBL and LL patients without reaction.

^bBL and LL patients with ENL.

BI, bacillary index in the lesion; max, maximum; min, minimum.

Cell culture

Neutrophils (1×10^6 total cells) were cultured in the absence or presence of 20 ng/ml of IL-10 (PeproTech) and/or in different concentrations (1, 10, or 50 $\mu\text{g/ml}$) of *M. leprae* γ -irradiated whole cells (NR-19329; BEI Resources) in 12-tissue-culture

well plates for 24 h and incubated at 37°C with 5% CO₂. Supernatants were then collected after being spun at $350 \times g$ for 5 min for use in ELISA.

The experiments comparing live and dead *M. leprae* were carried out with the bacteria kindly provided by J. Krahenbuhl (National Hansen's Disease Program, Laboratory Research Branch, Louisiana State University, Baton Rouge, LA) through the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). The bacteria were obtained from footpads of athymic *nu/nu* mice. A part of the *M. leprae* suspension was killed by irradiation using a method described elsewhere (12). Neutrophils were infected with live or stimulated with γ -irradiated (dead) *M. leprae* at multiplicity of infection of 10:1 organisms per cell and exposed for 24 h at either 33°C or 37°C in 5% CO₂, respectively. Cell extracts were used for gene expression analyses.

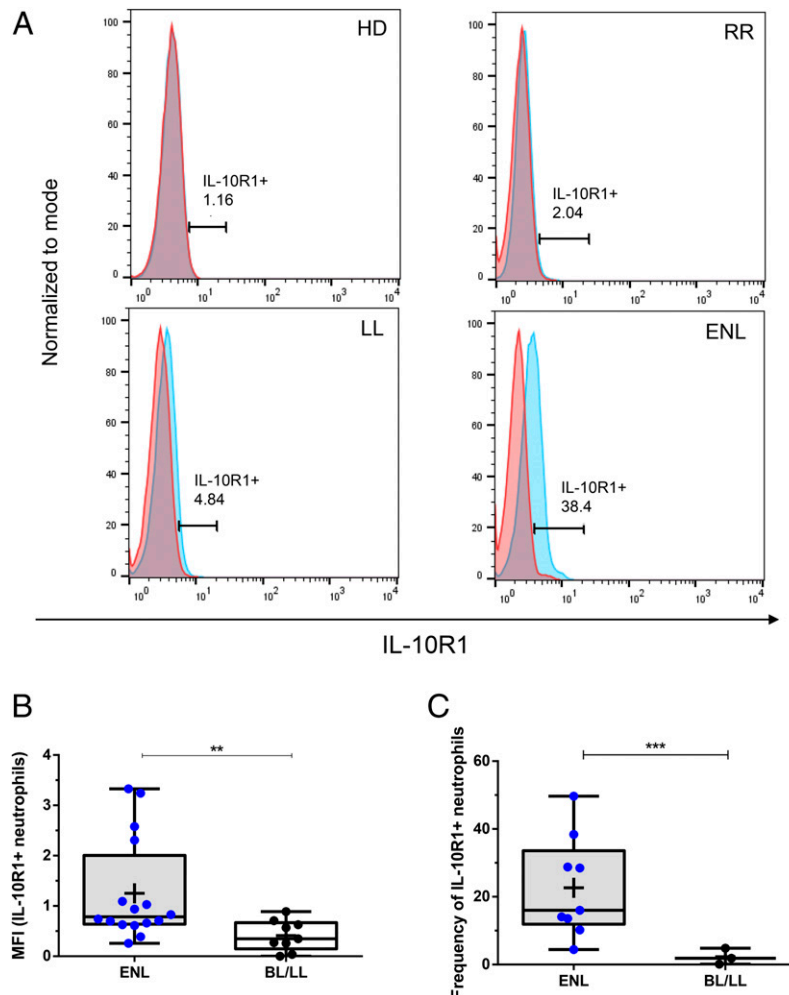


FIGURE 1. IL-10R1 is selectively expressed on circulating neutrophils during ENL.

(A) Representative histograms of IL-10R1–stained neutrophils of each group of participants: healthy donors (HD; $n = 21$), BL and LL patients without reaction (BL/LL; $n = 9$), and with ENL ($n = 19$) or RR ($n = 6$). Red histogram, control; blue histogram, IL-10R1 staining. (B) MFI values for each LL and ENL patients. MFI was calculated by subtracting the MFI of the immunocoujugated-stained neutrophils from the corresponding IL-10R1–stained cells. (C) Frequency of IL-10R1⁺ neutrophils. Box plots show median, interquartile range, sample minimum, and maximum. Dots represent individual patients. Statistic (B and C), ANOVA. +, mean. ** $p < 0.01$, *** $p < 0.001$.

Quantitative RT-PCR

Total RNA was isolated from neutrophils via the TRIzol reagent (Thermo Fisher Scientific) method according to the manufacturer's instructions. After isolation, RNA was treated with the DNA-free DNA Removal Kit (Thermo Fisher Scientific). Subsequently, RNA concentration was quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop) and integrity was evaluated by 1.2% agarose gel electrophoresis using a UV spectrophotometer. One microgram of total RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis SuperMix

(Thermo Fisher Scientific), also according to the manufacturer's suggestions. Quantitative RT-PCR was performed using the TaqMan designed primers: *CCL4* (*Hs99999148_m1*) and *IL-8* (*Hs00174103_m1*). Quantitative RT-PCR were run at a final volume of 10 μ l containing 10 ng of cDNA, TaqMan Fast Universal PCR Master Mix (2 \times) (Thermo Fisher Scientific) and 1 \times of each primer. All reactions were carried out in triplicate with appropriate controls (no reverse transcriptase-negative controls or template-negative controls) and incorporated into each run. Briefly, the reactions were performed on a StepOnePlus

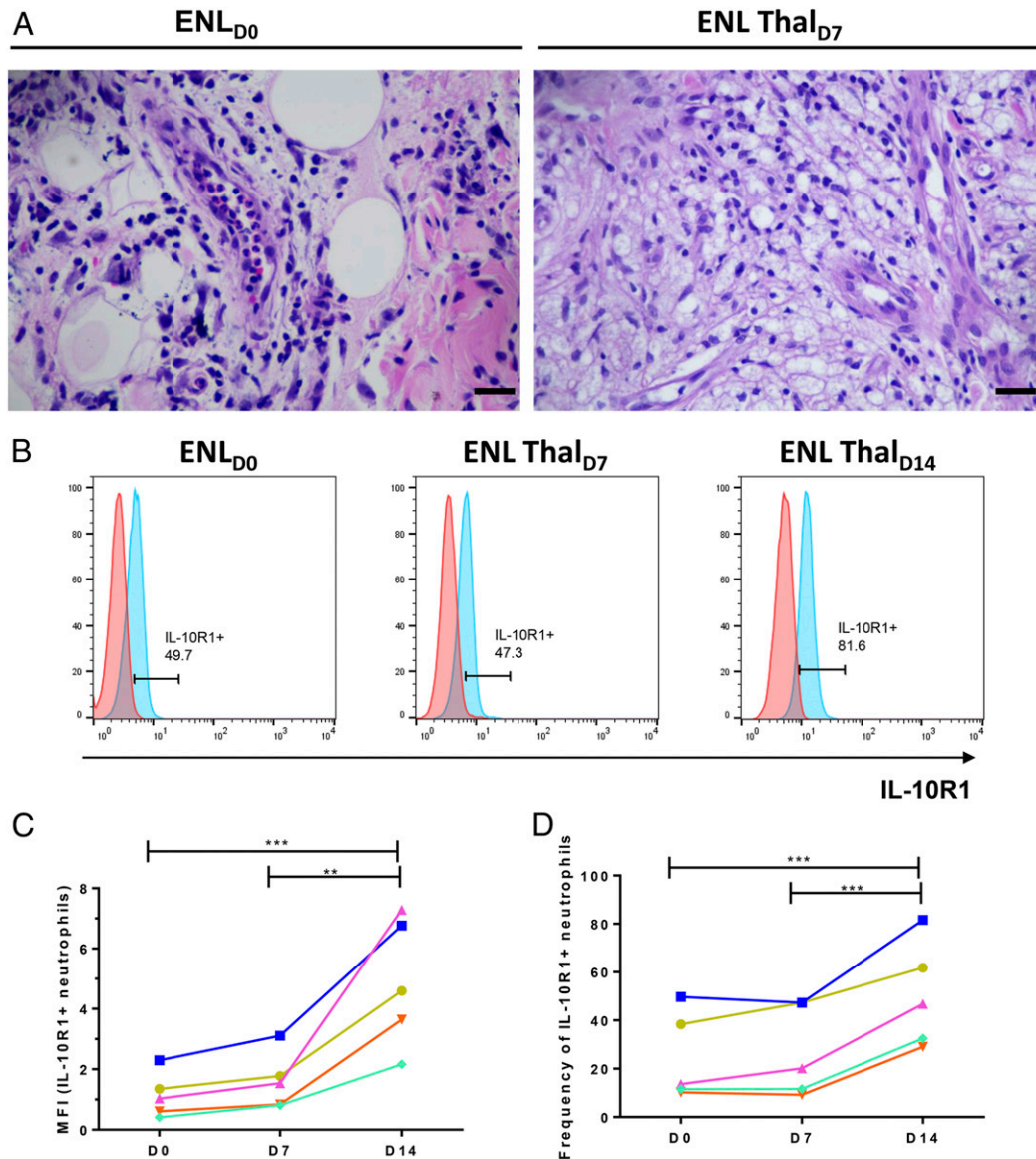


FIGURE 2. Upregulation of IL-10R1 on ENL neutrophils during thalidomide treatment.

(A) Two-week longitudinal follow-up of thalidomide-treated ENL patients. ENL skin lesions at the onset of diagnosis (ENL) and after 7 d of thalidomide treatment (ENL Thal_{D7}). Images are representative of one ENL patient ($n = 5$). Scale bar, 100 μ m. (B) Representative pattern of IL-10R1 expression in one ENL patients. D0, before treatment; D7, 7 d after starting treatment; D14, 14 d after starting treatment with thalidomide. (C) MFI values for each ENL patient examined for 2 wk. (D) Frequency of IL-10R1⁺ neutrophils for each ENL patient followed for 2 wk. Points and connecting lines represents individual patients. Statistic, repeated measure ANOVA. ** $p < 0.01$, *** $p < 0.001$.

Real-Time PCR Systems thermocycler (Applied Biosystems). Thermal cycling conditions comprised an initial incubation at 95°C for 20 s, 40 cycles of denaturation at 95°C for one sec, and annealing and extension at 60°C for 20 s. The relative expression of the genes of interest was normalized by GAPDH (Hs9999905_m1). The relative expression data were calculated using the $2^{-\Delta\Delta}$ cycle threshold method.

Cytokine determination

Concentrations of TNF, IL-1 β , IL-1RA, IL-6, IL-8, IL-10, TGF- β 1, and CCL4 in cell-free culture supernatants or serum samples were determined by using commercially specific ELISA kits (ELISA) that were processed according to the manufacturer's instructions. All assays were performed in duplicate. Results were expressed in picograms per milliliter after data processing using SoftMax Pro Software, version 4.8 (Molecular Devices). The IL-1RA ELISA Kit was purchased from PeproTech, and the other ELISA kits came from eBioscience (Thermo Fisher Scientific).

Immunofluorescence assay

Double immunofluorescence was performed on leprosy skin lesions and examined as previously described (7). In brief, Abs against IL-10R1 (Santa Cruz Biotechnology) and myeloperoxidase (Santa Cruz Biotechnology) along with their respective isotypes were incubated with tissue sections, followed by incubation with Alexa Fluor 532 goat anti-rat IgG and Alexa Fluor 633 goat anti-rabbit IgG-specific secondary Abs (Thermo Fisher Scientific). The nuclei were stained with DAPI (Molecular Probes), and the slides were mounted with VECTASHIELD Mounting Medium (Vector Laboratories). Tissues were imaged using an Axio Observer.Z1 fluorescence microscope equipped with a Colibri.2 illumination system (Carl Zeiss) and an EC Plan-Neofluar 40 \times /1.30 oil objective. Images were acquired via an AxioCam HRm digital camera and AxioVision Rel. 4.6 software (Carl Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software). Comparisons between more than two groups of not normally distributed data were performed by way of the Kruskal–Wallis and Dunn multiple comparison tests. Differences between the two groups were assessed by the two-tailed Student *t* test; the Wilcoxon test was used to compare paired samples, and the adopted statistical significance level was $p < 0.05$. The Spearman correlation coefficient was used to assess associations between the expressions of CD64 (polymorphonuclear neutrophil CD64 index) and IL-10R1 (median fluorescence intensity [MFI] value).

RESULTS

Patient clinical background

Twenty-eight patients with ENL reaction and 28 BL/LL patient controls without any reaction were recruited during the period covering 2009–2019. All ENL patients were untreated with

corticosteroid and/or thalidomide before recruitment. At time of recruitment, two ENL patients were previously untreated with MDT, nine are on MDT, and 17 were completed MDT treatment. All BL/LL patients were about to start MDT (Table I).

Identification of a distinct population of circulating IL-10R1⁺ neutrophils among ENL patients

To investigate the effects of ENL on the surface expression of IL-10R1 in circulating neutrophils, peripheral blood neutrophils from the whole blood of leprosy patients were isolated. As a first step, the purity of the neutrophils isolated from the peripheral blood was determined. Supplemental Fig. 1A depicts a flow cytometer dot plot showing the uniform CD16 expression in normal granulocytes. Microscopic analyses showed fewer contaminating eosinophils in the neutrophilic preparations (Supplemental Fig. 1). Regarding ENL patients, our results demonstrated that IL-10R1 expression was unique, as shown in the representative histograms in Fig. 1A. Our data also confirmed that healthy donor neutrophils do not express IL-10R1 (Fig. 1A). Analysis of the MFI revealed that ENL patients expressed higher levels of IL-10R1 than BL/LL (Fig. 1B). Moreover, the frequency of IL-10R1⁺ neutrophils was higher when compared with that found in BL/LL patients (Fig. 1C). These results, although displaying a variation among ENL patients, did not detect any correlations with either the severity of the clinical manifestations of ENL and IL-10R1 expression (MFI) or the frequency of IL-10R1⁺ cells. In this connection, our group previously showed that ENL neutrophils expressed CD64 (7), a marker of neutrophilic activation. It was then postulated whether neutrophilic CD64 expression correlated with IL-10R1 expression. In this context, the quantitative expression of CD64 was found to strongly correlate with that of IL-10R1 ($r = 0.9429$; $p = 0.0167$). The IL-10R1 expression was evaluated in neutrophils of BL/LL patients with RR, another leprosy immune reaction. RR neutrophils

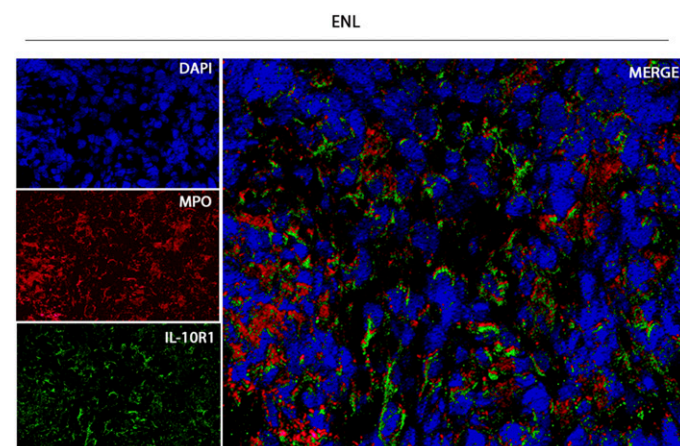


FIGURE 3. ENL lesions exhibit IL-10R⁺ neutrophils.

Representative image of immunofluorescence in histological sections of an ENL skin lesion before thalidomide treatment ($n = 3$; original magnification $\times 400$). Neutrophils were considered myeloperoxidase-positive (MPO⁺) cells. IL-10R1 (green), myeloperoxidase (MPO) (red), and nucleus (DAPI, blue).

express low levels of IL-10R1, and the frequency of IL-10R1⁺ neutrophils is also low (Fig. 1A). Both parameters resemble neutrophil levels found in healthy donors (Fig. 1A). The levels of IL-10R2 expression in the neutrophils of healthy donors, ENL, and BL/LL patients were quantitatively comparable (Supplemental Fig. 2).

The expression of IL-10R1 on the surface of circulating neutrophils increased during thalidomide treatment in ENL patients

A 2-wk follow-up of five ENL patients successfully treated with thalidomide revealed a regression of neutrophilic infiltrate in skin lesion (Fig. 2A). Interesting was the fact that IL-10R1 expression increased over the 2-wk covered (Fig. 2B). It was also observed that both MFI and the frequency of IL-10R1⁺ cells rose despite the introduction of thalidomide treatment (Fig. 2C, 2D).

IL-10R1⁺ neutrophils are present in ENL lesions

Considering that neutrophils are the signature cells in ENL lesions (13, 14), the expression of IL-10R1 in neutrophils presented in lesions was evaluated. The results revealed that neutrophils, the myeloperoxidase-positive cells, located in ENL lesions likewise expressed IL-10R1 (Fig. 3A).

Upregulation of IL-10R1 expression in neutrophils of ENL patients is functional

Because the responsiveness of neutrophils to IL-10 is conditioned by the surface expression of IL-10R1 (15), the ability of ENL neutrophils to directly respond to IL-10 in terms of TNF secretion was examined. ENL patients' neutrophils spontaneously released TNF (Fig. 4A). In contrast, no detectable TNF levels were observed in the supernatants of BL/LL neutrophilic cultures (Fig. 4A).

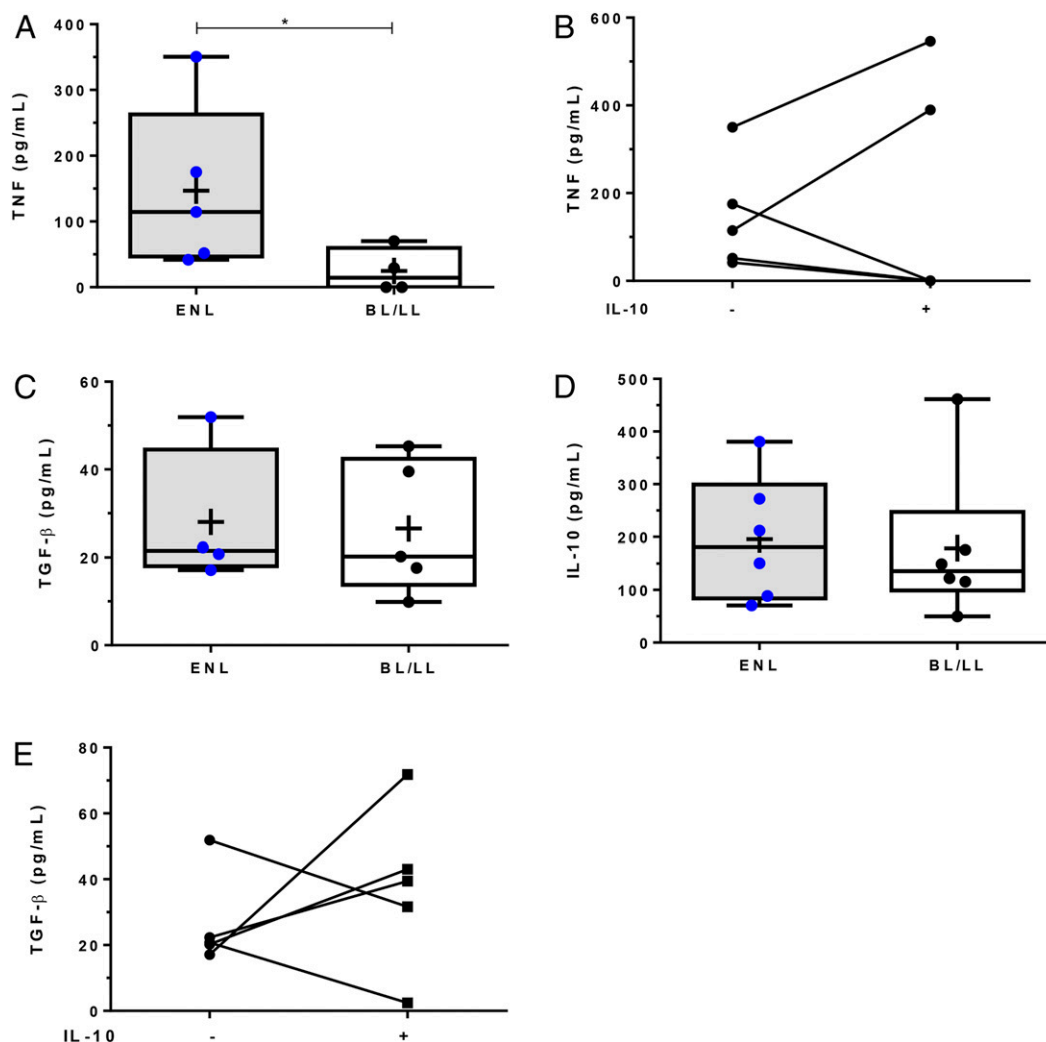


FIGURE 4. Effect of IL-10 on freshly isolated ENL neutrophils.

TNF (A and B), TGF-β (C and E), and IL-10 (D) spontaneous secretion in 24 h of cell culture of isolated ENL and BL/LL neutrophils. (B) TNF and (D) TGF-β levels in cell culture supernatants of ENL neutrophils treated or not with 20 ng/ml of IL-10. The cell-free supernatants were collected, and the cytokine levels were determined by ELISA. Box plots show median, interquartile range, sample minimum, and maximum. Points and connecting lines represents individual patients. Statistic, Mann-Whitney *U* test. +, mean. **p* < 0.05.

Besides, the addition of IL-10 to neutrophilic cell cultures decreased TNF secretion in three of the five ENL neutrophils (Fig. 4B), as was expected because of their IL-10R1 expression (Fig. 1A, 1B). Even so, two patients secreted TNF despite the addition of IL-10 (Fig. 4B). The spontaneous production of TGF- β and IL-10 levels by patient neutrophils was determined as well. There was, in the current study, no detectable difference between ENL and BL/LL neutrophils (Fig. 4C, 4D) in terms of TGF- β . The IL-10 addition induced higher levels of TGF- β in three of the five ENL neutrophils while inducing lower levels of TGF- β in two of the patient neutrophil cultures (Fig. 4E). Importantly, all ENL patients included in the study who received thalidomide had improvement in signs and symptoms regardless of the results of neutrophil cytokine secretion in vitro.

***M. leprae* upregulates IL-10R1 expression in neutrophils**

Previous studies have demonstrated that 4-h-cultured neutrophils express low levels of IL-10R1. However, adding LPS to those same cultures results in strongly upregulated IL-10R1 expression (9, 16). Because of the neutrophilic IL-10R1 expression in ENL patients and the fact that multibacillary patients are at a higher risk of developing ENL (17, 18), the ability of *M. leprae* to induce IL-10R1 expression in vitro was examined. To this end, highly purified, healthy donor neutrophils were stimulated in vitro with irradiated, dead *M. leprae* for 4 h. Cytometry analyses revealed that 4-h *M. leprae* neutrophils expressed significantly higher levels of IL-10R1 than unstimulated neutrophils (Fig. 5A, 5B).

IL-10 regulation of cytokines produced by *M. leprae*-stimulated neutrophils

Neutrophils from healthy donors were then purified and stimulated with different concentrations of dead *M. leprae*. Neutrophils stimulated in vitro by *M. leprae* produced IL-8, IL-1 β , IL-6,

IL-1RA, and CCL4 (Fig. 6C–G). Adding IL-10 to neutrophils previously cultured with *M. leprae* for 4 h both decreased the production of IL-8, IL-1 β , IL-6, IL-1RA, and CCL4 production (Fig. 6C–G) and increased TGF- β release (Fig. 6H). Neutrophils treated solely with IL-10 produced low TGF- β levels (Fig. 6H), confirming that the unstimulated neutrophils cultured for 4 h expressed low levels of IL-10R1. All together, these findings suggest that, in terms of cytokine release, *M. leprae* seems to groom neutrophils to achieve an optimal response to IL-10.

Inflammatory molecules are differentially regulated by dead versus live *M. leprae*

Whether or not live *M. leprae* was able to induce IL-8 and CCL4 in vitro was also examined. For this purpose, healthy neutrophils were infected or stimulated by live or dead *M. leprae*, respectively, in a multiplicity of infection of 10:1 (cell/bacteria). Our data suggested that the capability of dead *M. leprae* to induce IL-8 and CCL4 mRNA expression was superior to that of live bacteria (Fig. 7A, 7B). Indeed, IL-10 treatment reduced both expressions (Fig. 7A, 7B).

DISCUSSION

Neutrophils have long been considered a well-defined, homogeneous cell population. Nonetheless, the concept of the possible existence of neutrophilic subpopulations has emerged because of the demonstrated presence of circulating neutrophilic subsets in cancer and chronic inflammations (19).

In the current study, the unique presence of a neutrophilic subset expressing IL-10R1 during ENL as well as of IL-10R1⁺ neutrophils in the skin lesions of ENL patients prior to thalidomide treatment were investigated. A previous study by our group revealed that ENL patient neutrophils expressed CD64 (Fc γ RI)

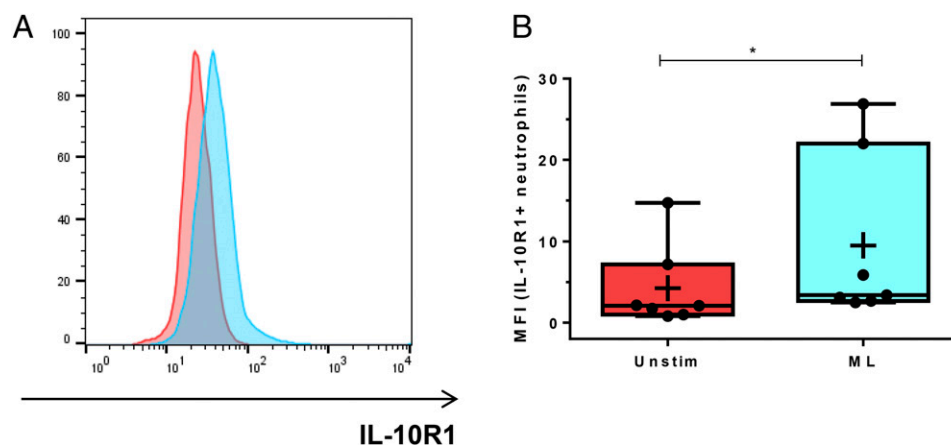


FIGURE 5. Effect of *M. leprae* on primary healthy neutrophils.

(A) Healthy neutrophils (1×10^6 total cells) were cultured for 4 h in the absence (unstimulated [Unstimu], red histogram) or presence of $10 \mu\text{g/ml}$ *M. leprae* (ML, blue histogram) were stained with anti-IL-10R1 and analyzed by flow cytometry. (B) MFI values for 4-h-Unstimu or *M. leprae*-stimulated neutrophils. Box plots show median, interquartile range, sample minimum, and maximum. Points represents individual patients. Statistic, Wilcoxon test. +, mean. * $p < 0.05$.

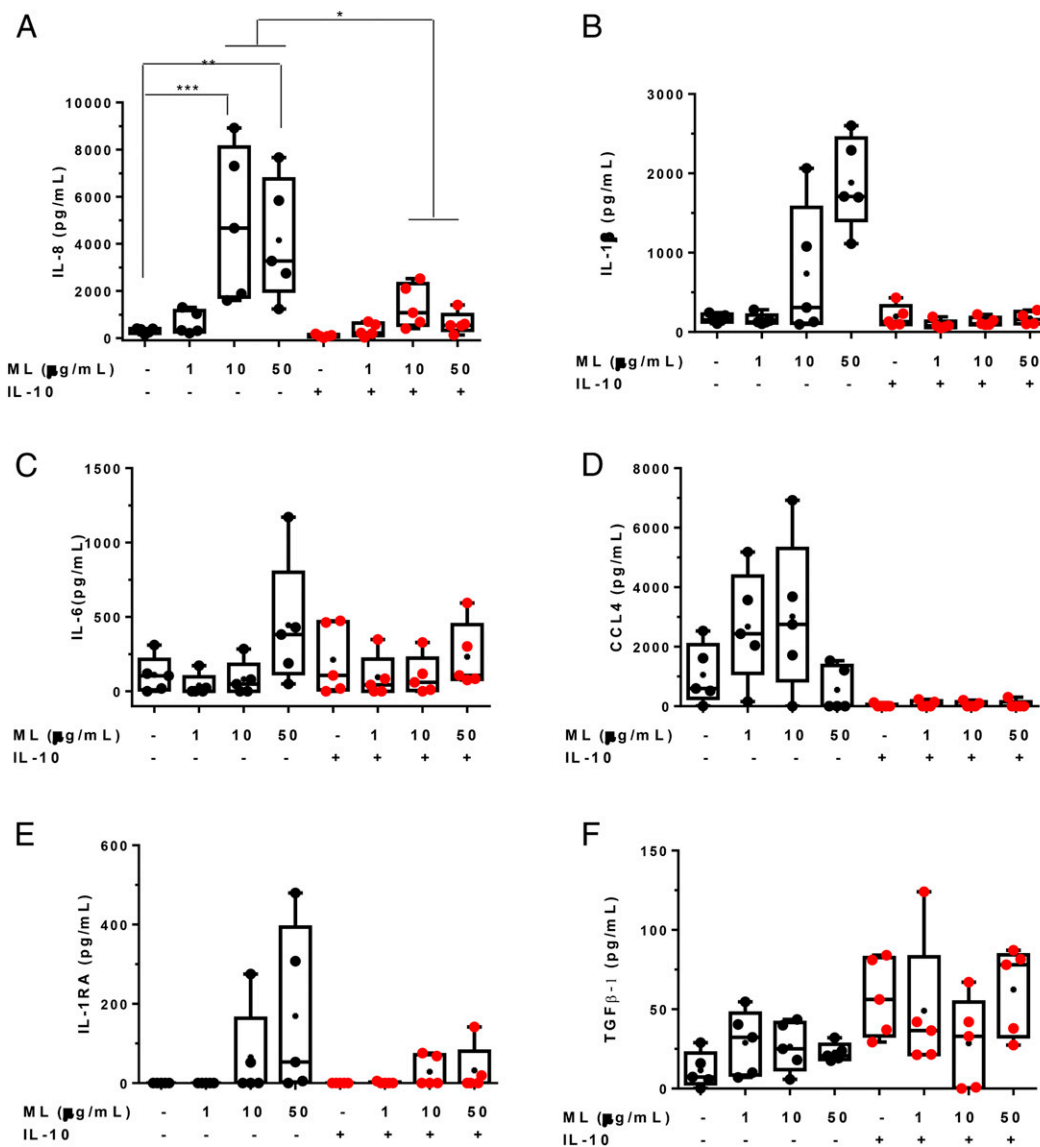


FIGURE 6. Effect of the addition of IL-10 on the ability of *M. leprae* to modulate cytokine release by neutrophils.

(A) IL-8, (B) IL-1β, (C) IL-6, (D) TGF-β1, (E) IL-1RA, and (F) CCL4 in cell-free culture supernatants of isolated neutrophils. Neutrophils (1×10^6 total cells) were incubated with dead *M. leprae* for 4 h and then cultured for 21 h after addition of 20 ng/ml of IL-10. The cell-free supernatants were collected, and the cytokine levels were determined by ELISA. Box plots show median, interquartile range, sample minimum, and maximum. Black points represent individual patients and red ones indicate the addition of IL-10 in neutrophil cultures. Statistics, ANOVA. +, mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ML, *M. leprae*.

(7), a marker of neutrophilic activation whose expression is correlated to ENL severity.

The present study showed that IL-10R1 expression in neutrophils also correlated to CD64. To our knowledge, this is the first work to point to the neutrophilic surface expression of IL-10R1 as a potential biomarker of ENL patients. One of the most desirable characteristics of a biomarker is that its expression becomes modified as the result of treatment. Our results pointedly demonstrated that despite the introduction of thalidomide treatment in ENL patients, IL-10R1 expression was increased in circulating neutrophils as well as the frequency of IL-10R⁺ cells. In contrast,

thalidomide treatment downregulated CD64 expression in blood neutrophils (7).

Thalidomide has been used to treat other immune-mediated diseases such as systemic lupus erythematosus, multiple myeloma, and inflammatory bowel disease (20). Thalidomide has an estimated efficacy of from 70 to 90% in ENL patients, who not only undergo a complete recovery after just a few days of treatment, but also have lower nodular numbers and regression of their neutrophilic inflammatory infiltrate (6, 7).

One of the proposed mechanisms that might elucidate the intensification of thalidomide activity is the reduction in TNF and

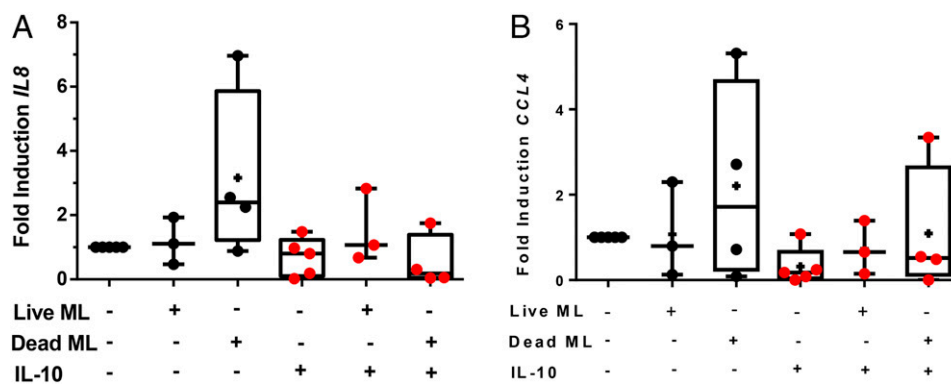


FIGURE 7. Effect of IL-10 on CXCL8/IL-8 and CCL4 mRNA expression induced by live or dead *M. leprae* in cultured neutrophils.

Fold change in expression of CXCL8/IL-8 (A) and CCL4 (B) in healthy neutrophils ($n = 4$) stimulated with (red dots) or without (black dots) IL-10. Neutrophils (3×10^6 total cells) were incubated with or without live or dead *M. leprae* multiplicity of infection (MOI) (1:10, neutrophil/bacteria) for 4 h and then cultured for 21 h after the addition of 20 ng/ml of IL-10. After incubation, neutrophils were lysed for total RNA extraction. The expression levels have been normalized to GAPDH for each sample. Box plots show median, interquartile range, sample minimum, and maximum. Points represents individual patients. +, mean; ML, *M. leprae*.

IL-1 β serum levels, which, on the contrary, are elevated during ENL (4, 21). Previous studies by our group demonstrated that LPS-stimulated leprosy neutrophils with or without ENL produced TNF after 6 h but reduced its production after the addition of thalidomide (11). Lee and colleagues (22) have reported that thalidomide performed regulatory activities during neutrophilic recruitment through three major pathways: 1) IL-1 β induction via Fc receptor and TLR2 activity, 2) endothelial cell activation and increased E-selectin expression, and 3) the increased expression of inflammatory mediators derived from neutrophils and monocytes/macrophages.

In other neutrophilic-mediated conditions, there is evidence that neutrophils contribute to the pathogenesis and worsening of diseases through alterations in phagocytic capacity, induction of endothelial damage, and production of proinflammatory molecules (23). This is the first time, to our knowledge, that circulating neutrophils of ENL patients have been shown to spontaneously release TNF *ex vivo*, thus corroborating their role as key cells in ENL immunopathogenesis. In contrast, BL/LL patient neutrophils without ENL did not release TNF *in vitro*. Interestingly, TNF secretion from ENL neutrophils became blocked after adding IL-10 *in vitro*. Direct IL-10 activity on ENL neutrophils is consistent with the functional expressions of both IL-10R1 and IL-10R2. An IL-10 regulatory effect on ENL neutrophils was verified because of an increase in the release of TGF- β release in four out of eight patients. Similarly, IL-10R1 neutrophilic expression and signaling were significantly higher in the neutrophils of septic patients (24). These combined results point to the IL-10/IL-10R pathway as a potential target for developing new therapies to effectively control the advent of ENL.

Neutrophilic involvement in ENL addressed by way of the bioinformatic analyses of microarrays revealed the upregulation of genes associated with neutrophilic recruitment and activation (22). Several studies point out that the production of cytokines by neutrophils may determine certain ongoing conditions (25). It has been observed, for example, that *M. leprae*, LPS, and lipoarabinomannan

stimulate LL patient neutrophils to secrete IL-8 regardless of ENL involvement (11). In this study, we demonstrated that healthy neutrophils stimulated by *M. leprae* *in vitro* produced large amounts of the well-established proinflammatory mediators (i.e., IL-1 β , IL-1RA, IL-6, IL-8, and CCL4) that are so critical to the systemic inflammation associated with ENL (3). However, most interesting was the fact that *M. leprae* was able to induce IL-10R1 expression in healthy neutrophils. Adding IL-10 to *M. leprae*-cultured neutrophils decreased the release of proinflammatory cytokines. This result is in agreement with the full expression of IL-10R, which triggers neutrophilic responsiveness to IL-10.

It is of major significance that dead *M. leprae* were shown to be much better equipped than live *Mycobacterium* to induce CCL4 and IL-8 gene expression in neutrophils. Indeed, *M. leprae*-loaded neutrophils and monocytes of multibacillary patients circulate in the blood freely with no sign of systemic inflammation (26). Our data implied that *M. leprae* may hamper the inflammatory machinery as an immune escape mechanism. Silva and colleagues (27) have also demonstrated that dead, but not live *M. leprae*, can induce autophagy in human monocytes and that live *Mycobacterium* can reduce the autophagic activation triggered by dead ones.

The present report also demonstrated that the peripheral blood neutrophils of ENL patients displayed measurable surface levels of IL-10R1, unlike the neutrophils of either BL/LL patients or healthy donors. Contrariwise, IL-10R2 expression levels, already remarkable in normal neutrophils (10), remained unchanged in ENL neutrophils. This event appeared to lead to a specific upregulation of IL-10R1 membrane levels taking place solely in ENL neutrophils. A comparative study would have been impossible.

It is unfortunate that the low number of patients examined in the current study did not allow for the definitive classification of IL-10R1 as an ENL marker. A much larger cohort of clinical cases would certainly provide more useful information that could more reliably determine if the detection of surface IL-10R1 in ENL

neutrophils could be used as a potential indicator of disease outcome. Our research more precisely highlighted neutrophils as key cells in the systemic inflammation associated with ENL while also signaling the IL-10 pathway as a molecular target to better understand reaction and develop effective protocols for future interventions.

DISCLOSURES

The authors have no financial conflicts of interest.

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