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Involvement of TNF-Producing CD8$^+$ Effector Memory T Cells with Immunopathogenesis of Erythema Nodosum Leprosum in Leprosy Patients

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Abstract. Type 2 reaction (T2R) or *erythema nodosum leprosum* (ENL), a sudden episode of acute inflammation predominantly affecting lepromatous leprosy patients (LL), characterized by a reduced cellular immune response. This possibly indicates a close relationship between the onset of T2R and the altered frequency, and functional activity of T lymphocytes, particularly of memory subsets. This study performed ex vivo and in vitro characterizations of T cell blood subpopulations from LL patients with or without T2R. In addition, the evaluation of activity of these subpopulations was performed by analyzing the frequency of these cells producing IFN-γ, TNF, and IL-10 by flow cytometry. Furthermore, the expression of transcription factors, for the differentiation of T cells, were analyzed by quantitative real-time polymerase chain reaction. Our results showed an increased frequency of CD8$^+$/TNF$^+$ effector memory T cells (T_{EM}) among T2Rs. Moreover, there was evidence of a reduced frequency of CD4 and CD8$^+$ IFN-γ–producing cells in T2R, and a reduced expression of *STAT4* and *TBX21*. Finally, a significant and positive correlation between bacteriological index (BI) of T2R patients and CD4$^+$/TNF$^+$ and CD4$^+$/IFN-γ$^+$ T cells was observed. Thus, negative correlation between BI and the frequency of CD4$^+$/IL-10$^+$ T cells was noted. These results suggest that CD8$^+$/TNF$^+$ T_{EM} are primarily responsible for the transient alteration in the immune response to *Mycobacterium leprae* in ENL patients. Thus, our study improves our understanding of pathogenic mechanisms and might suggest new therapeutic approaches for leprosy.

INTRODUCTION

Despite the drastic reduction in the number of leprosy cases since the implementation of multidrug therapy (MDT), in 1980, by the WHO, the disease still appears as a relevant public health concern in endemic countries, including Brazil, which reported more than 10% of new cases detected in 2016 around the world.[1] The skin and peripheral nerve lesions, traditionally found in leprosy, are associated with the tropism of *Mycobacterium leprae* both to macrophages and to Schwann cells, which, in turn, are major reservoirs of both the pathogen and of the immune response from hosts. If untreated, the injuries provoked by such a response usually lead to atrophies, paresis, face disfigurement, and even blindness.[2] Given the influence of genetic factors on the disease process and on the clinical course of leprosy, the genes that influence the pattern and intensity of immunological response are natural candidates to determine both the occurrence of the disease and the clinical outcome thereof.[3]

Leprosy presents several clinical manifestations ranging within a spectrum, and the most acceptable classification in scientific studies was provided by Ridley and Jopling,[4] in 1966, with the division of the disease into five forms, two of them being polar and the other three borderline forms. On one side, the tuberculoid pole (TT) is characterized by Th1-mediated immune response and a low number of skin lesions, with the appearance of granulomas, lymphocitic infiltrates, and, in unusual cases, bacilli. At the opposite end of the spectrum, the lepromatous pole (LL) is characterized by absent Th1 cellular immunity and high titers of antibodies against *M. leprae*, which are not effective in controlling the high load of bacilli. The nodular skin lesions disseminated throughout the body of LL patients present a high number of foamy macrophages and histiocytes containing high numbers of acid-fast bacilli with very few lymphocytes in a completely disorganized manner.[5,6]

The dynamic nature of the immune response to *M. leprae* leads to spontaneous fluctuations in the clinical state, which are known as leprosy reactions. As disclosed by a prior study performed by our group, more than half of the multibacillary patients are affected by acute inflammation episodes, known as leprosy reactions, either before, during, or after MDT, and even after healing.[7] The type 1 reaction (T1R), also referred to as reversal reactions, mostly affects borderline patients, as a cause of the increased frequency of antigen-specific CD8$^+$/CD45RA$^+$ T cells expressing the cutaneous leukocyte-associated antigen molecule, both in the blood and inside the lesions.[8] On the other hand, the type 2 reaction (T2R), also known as erythema nodosum leprosum (ENL), is characterized by a sudden exacerbation of the chronic conditions, with the appearance of additional skin lesions, reactivation of former ones, formation of painful nodules, general malaise, and aggravation of neurological damage. An early diagnosis of T2R, together with a suitable therapeutic management, may contribute to reduce the incapacities resulting from these episodes that are often severe.[9,10] Several decades ago, it was thought that the participation of immune complexes and the activation of complement components were associated with the genesis of T2R.[11] In addition, one study noted a modification in the proportion of CD4 and CD8$^+$ T cells in the skin lesions and blood from LL patients, either with or without reaction.[12] More recently, both pro- and anti-inflammatory mediators were shown to be increased in the plasma of T2R patients.[13] Nevertheless, one should still clarify the role of different T lymphocyte subsets in the secretion of these cytokines, as well as the way these antagonistic cytokines act in synergy to characterize the reactional episode and the severity thereof.

T lymphocyte subsets perform very heterogeneous functional activities and contribute in distinct forms to the dynamics of infectious diseases. Such subsets present differentiations

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according to their surface molecules. The identification of CCR7 and CD45RA is crucial for the characterization of T lymphocyte subsets.[14] The aforesaid two molecules are commonly used to classify the following T lymphocyte subsets: *naive* ($CCR7^+/CD45RA^+$), central memory ($CCR7^+/CD45RA^-$), effector memory ($CCR7^-/CD45RA^-$), and effector cells ($CCR7^-/CD45RA^+$).[15]

Therefore, this work intends to identify the cytokine (IFN-γ, TNF and IL-10)-producing T cell subsets, the expression of the transcription factors thereof, as well as to compare the relevant results with the clinical manifestations found in the genesis of T2R patients.

MATERIAL AND METHODS

Studied population. Forty-nine individuals were included, among whom 19 were LL patients with T2R (by the time of diagnosis of the first reactional episode and without use of immunosuppressant drugs, such as thalidomide or prednisolone) and 15 were LL non-reactional patients. All patients were diagnosed according to Ridley and Jopling[4] criteria and accompanied at the Leprosy Outpatient Unit-FIOCRUZ. We also used blood samples from 15 healthy volunteers (HVs) with the same socioeconomic background as the patients and living in Rio de Janeiro city, which is known to be endemic for leprosy, the same area where the patients live too. None of the patients included in our work was below the age of 18, nor any other affected by acute or chronic infectious comorbidities were included in this study.

Ethical considerations. The study was approved by the Research Ethics Committee of the Oswaldo Cruz Foundation-FIOCRUZ (permit protocol number 518/09A) and a written informed consent was obtained from all individuals before specimen collection. For the sake of privacy and well-being of the studied individuals, we refrained from disclosing their identity (Table 1).

Peripheral blood mononuclear cells (PBMCs) collection and culture and in vitro stimulation assays. Peripheral blood mononuclear cells were obtained under endotoxin-free conditions from heparinized venous blood of patients and healthy donors in Ficoll-Hypaque (GE Healthcare AB, Uppsala, Sweden) density centrifugation. After a separation, part of freshly isolated PBMC were resuspended at 5×10^5/well in phosphate-buffered saline (PBS) for ex vivo analysis and the remaining were cultured in AIM V (Gibco BRL, Gaithersburg, MD) at 1×10^6/well of cultured PBMC for 6 hours in 96-well U-bottom culture plates (Costar, Cambridge, MA) in the presence of 1 μg/mL anti-CD28 and anti-CD49d (BD Biosciences, San José, CA). Then, the cells were stimulated with enterotoxin B (1 μg/mL) from *Staphylococcus aureus* (SEB; Sigma, St. Louis, MO), as a positive control for the viability of the T cells. As antigenic stimulation, 20 μg/mL of irradiated and sonicated armadillo-derived *M. leprae* (ML; supplied under the agreement NIH/NIAID contract N01 AI-25469 with Colorado State University, Fort Collins, CO) was used. For the assays of intracellular cytokines detection, the cultures were kept at 37°C with 5% CO_2 and 70% humidity, and during the last 2 hours, protein transport inhibitor brefeldin A (10 μg/mL; Golgi Plug; BD Biosciences) was added. The kinetics of responses to *M. leprae* and SEB were previously determined in HVs, reaching a peak at 6-hour cultures.

Analysis of surface molecules and intracellular cytokines on $CD4^+$ and $CD8^+$ T subsets by flow cytometry. All PBMC (ex vivo or in vitro) were resuspended in PBS (Gibco), 0.02% ethylenediaminetetraacetic acid (Sigma), and stained with 4',6-diamidino-2-phenylindole (DAPI) (Live/Dead Kit; Invitrogen, Grand Island, NY) for separation of dead cells, according to the manufacturer's instructions. Briefly, PBMC were incubated with DAPI (Invitrogen) for 30 minutes, washed by centrifugation, resuspended in flow cytometry buffer (PBS with 1% fetal calf serum [FCS] and 0.01% sodium azide, all from Gibco), and incubated for 30 minutes at 4°C, with surface monoclonal antibodies anti-CD3 V500, anti-CCR7 PerCp, anti-CD4 or anti-CD8 APC, anti-CD45RA Alexa Fluor 488 (all 1:50 dilution; Biolegend, San Diego, CA). Appropriate isotype controls (BioLegend) were used in all analyses. Then, PBMC were resuspended in 1% paraformaldehyde (PA; Sigma) and incubated for 30 minutes at 4°C. Six-hour cell cultures were

TABLE 1
Identification of study population

ID	Form of leprosy	Reaction	Gender	Age (years)	BI	DG
RE01	LL	T2R	F	23	4.0	0
RE02	LL	T2R	M	74	4.0	2
RE03	LL	T2R	M	42	4.5	1
RE04	LL	T2R	M	64	2.5	2
RE05	LL	T2R	M	32	2.5	1
RE06	LL	T2R	M	54	4.0	1
RE07	LL	T2R	M	66	3.75	0
RE08	LL	T2R	M	60	1.75	2
RE09	LL	T2R	M	18	3.75	2
RE10	LL	T2R	F	38	5.75	1
RE11	LL	T2R	F	57	4.5	1
RE12	LL	T2R	F	33	3.5	0
RE13	LL	T2R	M	26	3.5	0
RE14	LL	T2R	M	32	4.5	1
RE15	LL	T2R	M	45	2.5	0
RE16	LL	T2R	M	62	4.5	0
RE17	LL	T2R	M	33	4.5	1
RE18	LL	T2R	M	35	4.75	1
RE19	LL	T2R	F	27	5.0	1
LP01	LL	–	M	26	5.0	2
LP02	LL	–	M	32	5.0	0
LP03	LL	–	M	33	4.75	0
LP04	LL	–	M	39	5.0	0
LP05	LL	–	F	31	5.0	0
LP06	LL	–	F	31	4.75	0
LP07	LL	–	M	32	5.75	0
LP08	LL	–	M	24	5.0	2
LP09	LL	–	F	27	5.5	1
LP10	LL	–	M	38	5.5	0
LP11	LL	–	M	37	5.5	0
LP12	LL	–	F	40	5.0	1
LP13	LL	–	M	65	4.75	0
LP14	LL	–	M	37	5.0	0
LP15	LL	–	M	48	5.0	0
HV01	HV	–	M	22	–	–
HV02	HV	–	F	28	–	–
HV03	HV	–	F	50	–	–
HV04	HV	–	F	23	–	–
HV05	HV	–	F	26	–	–
HV06	HV	–	F	22	–	–
HV07	HV	–	F	40	–	–
HV08	HV	–	M	35	–	–
HV09	HV	–	M	40	–	–
HV10	HV	–	M	45	–	–
HV11	HV	–	F	22	–	–
HV12	HV	–	F	21	–	–
HV13	HV	–	F	27	–	–
HV14	HV	–	M	48	–	–
HV15	HV	–	F	22	–	–

BI = bacteriological index; DG = disability grade; F = female; HV = healthy volunteer; ID = randomized code for each patient or HVs to safeguard their identity; LL = lepromatous leprosy; M = male.

resuspended in 1:10 permeabilization buffer (PERM-2; BD Biosciences) and incubated for 10 minutes at room temperature. After this period, PBMC were washed by centrifugation and resuspended in flow cytometry buffer. Then, PBMC were stained with monoclonal antibodies for intracellular cytokines anti-IFN-γ PE-Cy7, anti-TNF Alexa700, anti-IL-10 PE, and their respective isotype controls (BioLegend) for 30 minutes at 4°C (Supplemental Table 1). After other washes by centrifugation, PBMC were resuspended in 1% PA (Sigma). The cells were acquired on a FACSAria (with DIVA Software; BD Biosciences); 50,000 events/sample were acquired within the lymphocyte region for the ex vivo analysis and 100,000 events/sample for the cultures. For flow cytometric analysis, the software Flowjo v. 7.5 (Tree Star Inc., Ashland, OR) was used.

PAXgene whole-blood RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). Whole blood obtained by venous puncture was stored in PAXgene tubes (BD Biosciences) at –80°C, for a period of less than 6 months. Whole RNA was prepared using PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified on a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). cDNA synthesis was carried out using the Superscript III RT-PCR kit (Applied Biosystems, Branchbug, NJ). TaqMan real time PCRs were performed via the TaqMan Fast Universal PCR Master Mix (2×) and specific primers and probes (Applied Biosystems). Briefly, PCR was performed in the StepOnePlus Real-Time PCR Systems (Applied Biosystems) at 95°C for 20 seconds, 40 cycles of 95°C for 1 second, and 60°C for 20 seconds. The studied genes were *TBX21* (Hs00203436_m1), *GATA3* (Hs00231122_m1), *RORC* (Hs01076122_m1), *STAT3* (Hs00374280_m1), *STAT4* (Hs01028017_m1), *STAT6* (Hs00598625_m1), and *FOXP3* (Hs01085834_m1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1) was used as a reference gene and mRNA was quantified using the $2^{-\Delta Ct}$ method (ΔCt = Ct [target gene] – Ct [endogenous gene]). Quantitative polymerase chain reaction conditions were the same as described previously for the gene expression analysis (Applied Biosystems).

Statistical and graphic analysis. The data were analyzed using GraphPad Prism 6.0 (GraphPad, San Diego, CA). The results are reported as % of median ± standard error of the median for ex vivo, and range and quartiles (25^{th} and 75^{th} percentile) for data referring to cytokine-producing T cells. To determine differences between stimulated (ML or SEB) and unstimulated cells conditions the Mann–Whitney test to group comparisons was used, as well as the Wilcoxon test for a correlation analysis. The adopted statistical significance level was $P < 0.05$.

RESULTS

Demographic and clinical analysis of the studied population. First, the sociodemographic and clinical data of all patients (ENL and LL) and HV group were compared. The ENL group presented a mean age of 43.2 ± 16.7 years, whereas in the LL group the mean age was of 36 ± 9.7 years, and in the HVs, the mean age was 31.4 ± 10.2 years. As to the gender, the reactional group comprised 64.3% male and 35.7% female, whereas the LL group consisted of 73.3% male and 26.7 female, and the HVs were 33.3% male and 66.7% female. In relation to the bacteriological index (BI) from T2R group, at the onset of the reaction, this group presented a BI of 3.88 (ranging between 1.75 and 5.75). Lepromatous leprosy group presented a mean BI of 5.1 (ranging from 4.75 to 5.75). The lepromin skin test (Mitsuda reaction) was negative in all the patients (data not shown). About 50% of the patients already presented sensibility loss or disabilities resulting from leprosy. This was disclosed by means of the assessment of functional disability grade (DG) performed in all patients. Among the T2R patients, 76.5% presented DG ≥ 1, with a mean of 0.9 (ranging between 0 and 2), whereas 26.6% of LL patients presented DG ≥ 1, with a mean of 0.4 (ranging between 0 and 2). All the patients received MDT, and the T2R patients were treated with thalidomide or prednisolone (Table 1).

Ex vivo and in vitro phenotypic characterization of PBMC from patients and HVs. The Supplemental Figure 1 describes the strategy for our analysis. The ex vivo assessment of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T lymphocytes from LL group with or without T2R, as well as from HV group disclosed an approximate 2:1 ratio for CD4/CD8 among LL patients, either reactional or not (Figure 1A and E). As to the T cell subsets, the frequency of T naive was decreased among T2R patients, when compared with LL ($P < 0.01$ in $CD4^+$ and $P < 0.001$ in $CD8^+$). Both, central and effector, memory subsets presented a significantly increased frequency among T2R. As to $CD4^+$, when compared with the LL group, we noted significant differences in the central memory ($P < 0.0001$) and effector memory ($P < 0.01$; Figure 1B) subsets. As to the $CD8^+$ T lymphocytes, the two groups of patients also presented significant differences for the two memory subsets ($P < 0.001$; Figure 1F). Although T2R presented higher frequency of memory T lymphocytes, the effector T $CD4^+$ subset appears to be reduced, when compared with LL and HVs (T2R versus LL, $P < 0.01$, and ENL versus HVs, $P < 0.001$). The in vitro analysis (ML and SEB stimulated cultures) discloses that, regardless of the presence of reaction, ML was unable to alter the frequency of T $CD4^+$ and $CD8^+$, either in their whole cells or in their subsets. Nevertheless, the antigen was able to increase the frequency of whole $CD4^+$ and $CD8^+$ T cells among HVs ($P < 0.01$; Figure 1C and G). The results relating to the frequencies of T lymphocyte subsets from T2R and LL, either in the presence of ML stimuli or not, are closely similar to the ones from the ex vivo analysis, with higher frequencies of central and effector memory cells among T2R, in addition to higher frequencies of naive and effector T lymphocytes among LL (Figure 1D and H). In all the cultures, SEB, used as control, appeared to be positive (data not shown).

Profile of cytokine-producing $CD4^+$ and $CD8^+$ T lymphocyte subsets. According to the strategy for the assessment of T lymphocyte subsets (Supplemental Figure 1), we started analyzing each of these subsets as to the production of TNF, IFN-γ, and IL-10 cytokines, traditionally associated with the immunopathogenesis of T2R. For such, we analyzed the frequency of cytokine-producing T cells in each region of specific subsets (Figure 2A and C). Initially, as expected, T2R presented decreased frequency of IFN-γ–producing $CD4^+$ and $CD8^+$ T lymphocyte subsets, when compared with LL and HVs ($P <$ 005; Figure 2D and G). This same group presented a significantly increased frequency of IL-10–producing $CD4^+$ and $CD8^+$ T cells, particularly in the naive and central memory subsets ($P < 0.05$; Figure 2F and I). As to the

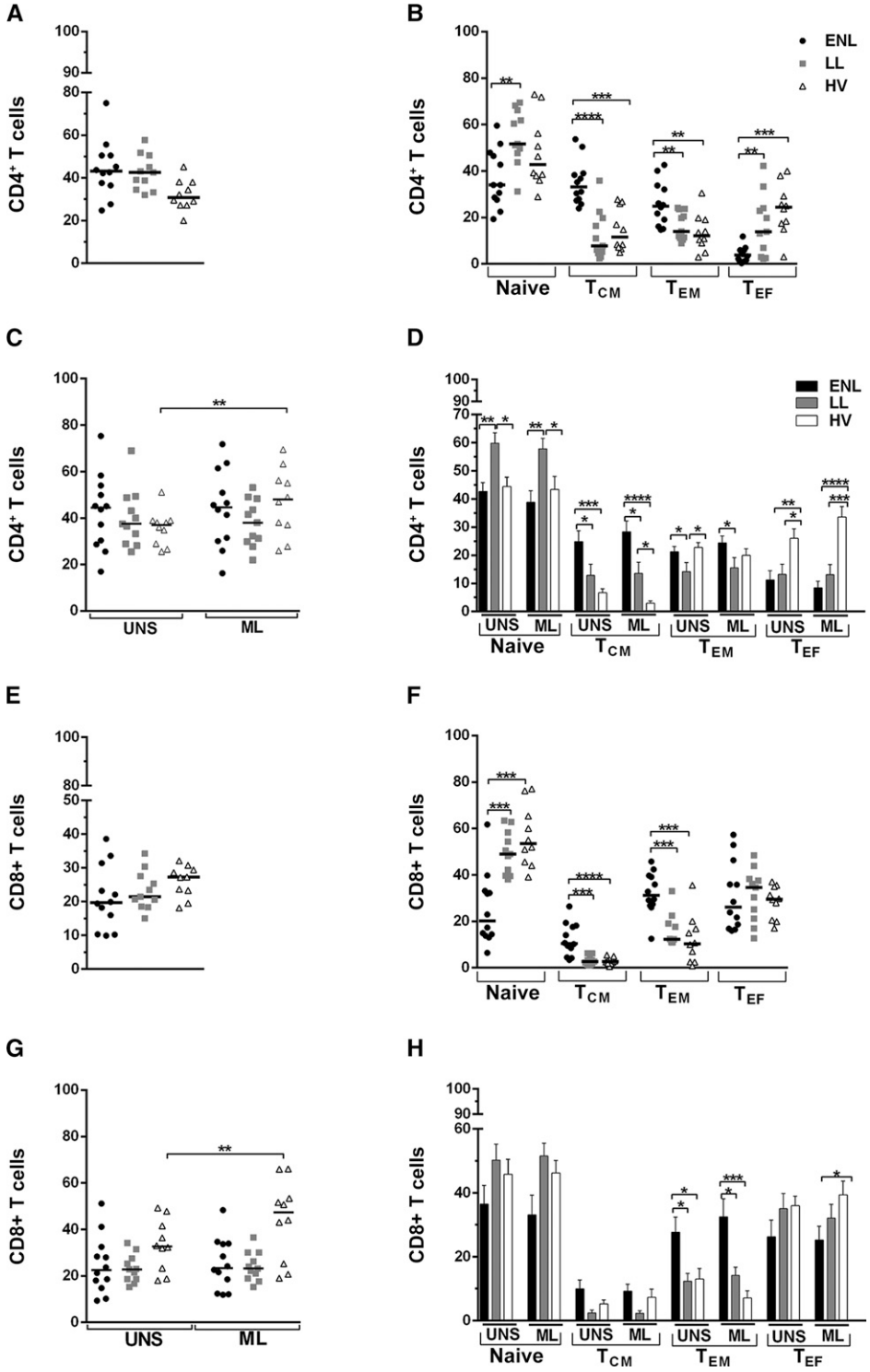

FIGURE 1. Ex vivo and in vitro phenotypic characterization of blood leukocytes from patients and healthy volunteers (HVs). In ex vivo analysis, the dot plots represent the groups, while the horizontal bars the mean of each group for whole cells CD3/CD4$^+$ (**A**) and CD3/CD8$^+$ (**E**). T lymphocyte subsets are shown under the same conditions (**B** and **F**). Response to ML from CD4$^+$ (**C**) and CD8$^+$ (**G**) T cells in 6-hour culture (UNS = unstimulated). The T lymphocyte subsets obtained in response to ML are shown in the set of bars with standard error deviation (**D** and **H**). The responses obtained on stimulation with SEB were positive for all the groups, and do not appear on the figure. Mann–Whitney and Wilcoxon statistical analysis, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. ENL = erythema nodosum leprosum; LL = lepromatous leprosy; HV = heathy volunteers; T_{CM} = central memory T cells; T_{EM} = effector memory T cells; T_{EF} = effector T cells.

frequency of TNF-producing cells, ML did not significantly alter the frequencies of some subsets of this cytokine (Figure 2E and H), despite the highly significant differences between the groups as to the effector memory T cells (T_{EM}) subset. As happened in the prior experiment, all the culture provided positive results on stimuli with SEB (data not shown).

Expression of T lymphocytes transcription factors associated to the differentiation of naive T lymphocytes. To

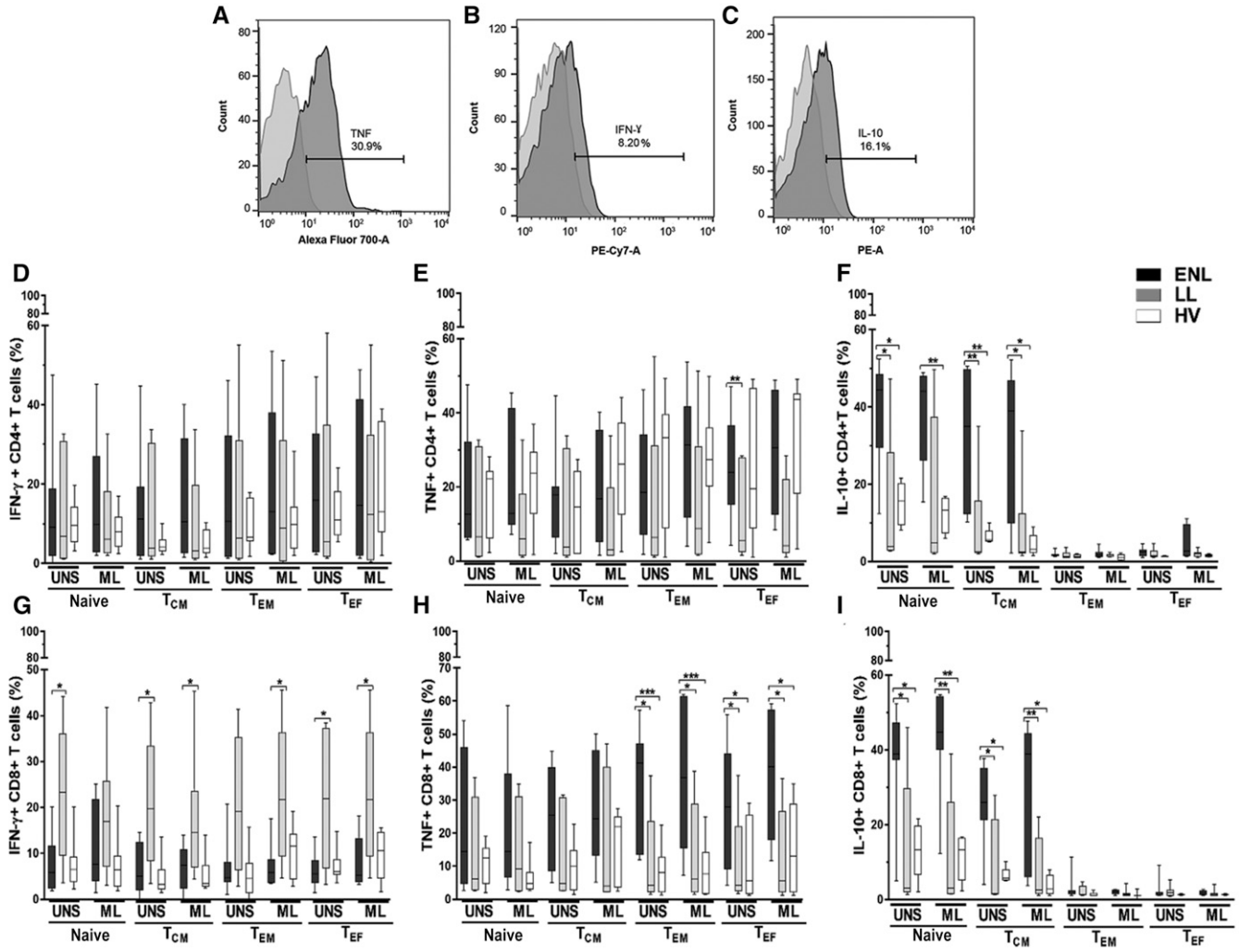

FIGURE 2. Profile of cytokine-producing $CD4^+$ and $CD8^+$ T lymphocyte subsets. Cytokine histograms show the way of acquisition of frequency values for each T lymphocyte subset (as shown in the strategy of analysis from the prior figure; **A**–**C**). The upper charts disclose the results obtained among the IFN-γ– (**D**), TNF– (**E**), and IL-10–producing $CD4^+$ T cell subsets (**F**). The lower charts disclose IFN-γ (**G**), TNF (**H**) and IL-10–producing $CD8^+$ T cells (**I**). Data obtained by multiparametric flow cytometric analysis with responses to ML from all the groups shown on the boxes, where the horizontal line and the vertical bars represent the mean frequencies and the distribution of intervals from each group assessed respectively on 6-hour culture. SEB as control were positive for all the groups (data not shown). Mann–Whitney test were used for the statistical analysis of group comparisons, being $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. UNS = unstimulated.

check any further alteration in the expression of T cells transcription factors among the groups studied herein, we assessed the gene expression with primer pairs for Th1, Th2, Th17, and T regulatory cells. The gene pair *TBX21* and *STAT4* was significantly reduced among T2R, when compared with LL and HV groups ($P < 0.01$; Figure 3A). In relation to the other gene pairs, no significant difference was noted among the studied groups. However, there were significant differences between T2R and LL groups with HVs. Healthy volunteers presented a significantly higher expression of genes associated with the differentiation for Th2 profile ($P < 0.05$; Figure 3B), in addition to higher expression of *RORC*, associated to the differentiation for Th17 profile ($P < 0.05$; Figure 2C). There was no significant difference as to the expression of genes relating to Treg (Figure 3D).

Correlation between the frequency of cytokine-producing $CD4^+$ and $CD8^+$ T lymphocytes and bacillary load among T2R. To investigate a correlation between clinical and immunological data, we associated the BI from patients and the results concerning the functional action of T cells. There was a significantly strong correlation between the frequency of IFN-γ-, TNF-, and IL-10–producing $CD4^+$ T lymphocytes and BI among T2R patients (Figure 4A–C). The correlation coefficient (r) between the frequency of T $CD3^+/CD4^+/IFN\text{-}\gamma^+$ was 0.8049 ($P = 0.0069$), whereas with $CD3^+/CD4^+/TNF^+$ this coefficient reached 0.8379 ($P = 0.0036$). By contrast, there was a significant negative correlation coefficient ($r < 0$) between the frequency of $CD3^+/CD4^+/IL\text{-}10^+$ T lymphocytes and BI among T2R patients, with $r = -0.817$ ($P = 0.005$; Figure 4C). There was no significant correlation coefficient, either positive or negative, between the frequency of cytokine-producing $CD8^+$ T cells and BI among T2R (Figure 4D–F).

DISCUSSION

Type 2 reaction or ENL appears as the most frequent complication from LL—affecting about a half of LL patients—and cause of severe sequelae, as its manifestation is systemic,

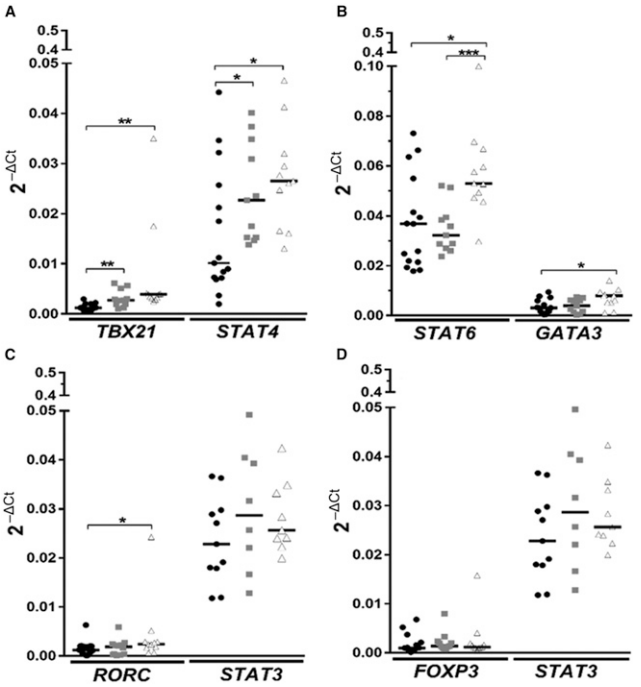

FIGURE 3. Assessment of the transcription factor gene expression inducing the differentiation of T lymphocytes. Assessment of *TBX21/STAT4* (**A**), *STAT6/GATA3* (**B**), *RORC/STAT3* (**C**) and e *FOXP3/STAT3* (**D**) through quantitative real-time polymerase chain reaction. The results are represented by $2^{-\Delta Ct}$ (being ΔCt the difference between Ct from the of interest gene and the reference gene) of experiments involving whichever group, where $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (Mann–Whitney test, group comparisons). In the figure, each dark circle represents a type 2 reaction patient, each grey square represents a LL patient, and each open triangle represents a healthy volunteers.

being able to provoke injuries in several organs, such as coagulation disorders and kidney failure, and may lead patients to death.[7,16] Thus, it is crucial to study the immune mechanisms triggering reactional episodes, as presented in this work, not only for the identification of the cell subsets involved with the genesis of these processes, but also for the detection of biomarkers of reactions, as well as contributing to an earlier clinical management and to the reduction of reactional morbidity.

In this way, our study intended to reach deeper clarifications on the participation of T cells in the immunopathology of T2R. Despite the lack of conclusive demonstration on which physiological alterations trigger this reactional type, several factors appear to contribute to triggering T2R episodes, including hormonal alterations, such as lactation, pregnancy, menopause, and puberty, in addition to other factors relating to the immune system of the hosts, such as stress, coinfections, and vaccination.[17] Gender was not proven to influence the development of T2R or of leprosy per se. Of note, however, about three quarters of the patients studied herein were male, and the same proportion presented T2R. Notwithstanding the changes in the social and economic profile in Brazil, particularly throughout the last three decades, with a higher number of women entering into the job market and having more social interactions, most individuals affected by the disease are still of male gender.

As disclosed by several studies, a high BI still appears as a risk factor for T2R. These data demonstrated that lepromatous patients with BI ≥ 4 present 39% higher risk of developing T2R, when compared with patients with lower BI.[18,19] Thus, the patients assessed herein actually presented a mean BI of 4.73 at the diagnostic onset, before starting MDT, and were included into the risk group for T2R.

The first step toward characterization of the participation of T cell subsets in the genesis of T2R consisted in assessing the frequencies of CD4 and $CD8^+$ T cells. Lepromatous leprosy patients, either reactional or not, showed a 2:1 frequency ratio of CD4/CD8. However, HVs curiously showed a 1:1 ratio of CD4/CD8, in addition to an alteration in the frequency of whole T cells, after in vitro stimulation by *M. leprae*. The healthy volunteers group disclosed an increased frequency of CD4 and $CD8^+$ T lymphocytes, although the CD4/CD8 ratio remained unaltered. The ratio 1:1 CD4/CD8 among HVs seems to be associated to the fact that Brazil is still endemic for leprosy, so that these individuals, who are continuously exposed to *M. leprae* since their birth, present PBMC with substantial levels of IFN-γ on stimulation by *M. leprae*.

Among the patients, the presence of *M. leprae* in the culture did not give rise to alterations in the frequency of whole T cells, either $CD4^+$ or $CD8^+$. Such a fact is possible because of the high bacillary load previously presented by these patients, which made the in vitro stimulation with *M. leprae* antigen irrelevant. Moreover, one should bear in mind the high number of lipids inside the bacterial cell wall, which probably hide the protein antigens and appear as an obstacle for the differentiation of T cells.[20]

Thus, the higher frequency presented by T memory subset cells (both central and effector), either for $CD4^+$ or $CD8^+$ phenotype in T2R, may be attributed to the exposure of new antigens released by *M. leprae*, due to intense fragmentation of bacilli during MDT. This hypothesis is corroborated by the fact that non-reactional LL patients studied herein had not started MDT, besides presenting a higher frequency of effector $CD4^+$ T lymphocytes, when compared with T2R patients who had undergone average 8-month anti-leprosy treatment.

Indeed, the frequency of antigen-specific memory T cells presents a differential dynamic varying not only according to the pathogen load, to the treatment, but also to the kind of response from hosts. In a recent work, Axelsson-Robertson and collaborators disclosed that memory T cells, particularly central memory $CD8^+$ T lymphocytes, underwent a gradual reduction during treatment of patients affected by the active form of tuberculosis.[21] In addition, as shown by another study, patients affected by acute tuberculosis presented a significantly reduced frequency of effector $CD8^+$ T cells, either before or during treatment, when compared with individuals diagnosed with latent infection. These results suggest the relevance of such subset in the resistance to *Mycobacterium tuberculosis*.[22] Moreover, a study with volunteers with a history of cutaneous leishmaniasis and patients with the disease, showed that circulating $CD8^+$ T_{EM} population were responsible for Leishmania-induced IFN-γ production in human cutaneous leishmaniasis.[23]

Despite the very different actions performed by these subsets, a recent work by Negera et al.[24] demonstrated the participation of memory T cell in the onset of T2R, and throughout the clinical follow-up of the disease, these data reported showed an increase of total memory T cells in untreated ENL patients. However, the authors failed to assess

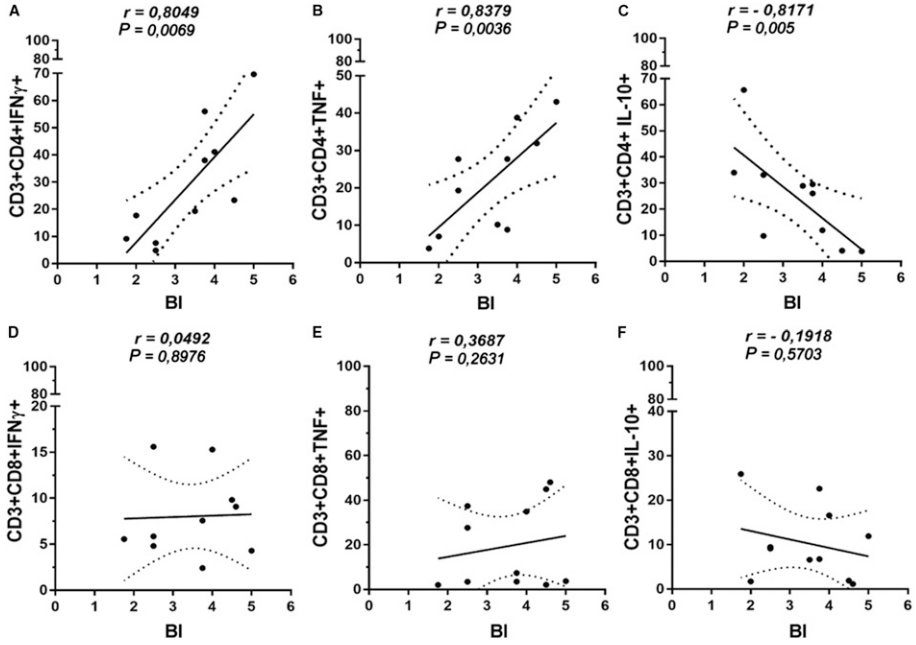

FIGURE 4. Correlation between the frequency of cytokine-producing $CD4^+$ and $CD8^+$ T lymphocytes and bacillary load among type 2 reaction (T2R). Correlation between the BI and the frequency of IFN-γ– (**A** and **D**), TNF– (**B** and **E**). and IL-10 (**C** and **F**)–producing $CD4^+$ and $CD8^+$ T cells among T2R. Each dot represents an individual, the correlation coefficients are shown in the lines (*r* values) and the dashed lines show the intervals with 95% confidence. Assessment through the Spearman method with significance values shown in *P*.

the inflammatory mediators produced by these subsets, and only admitted their participation in the reactional episode. So, there may be a close relationship between the genesis of T2R and alterations in the frequency and functional activity of T cells, particularly inflammatory cytokine-producing memory subsets.

In our in vitro analysis, on stimulation with *M. leprae*, the four studied subsets kept the same frequency pattern, although the relevant fact was once more the high frequency of memory cells among T2R. According to others, T2R patients, when compared with non-reactional LL patients, presented high IL-7 plasma levels.[13] As IL-7 is a cytokine primarily produced by fibroblasts and bone marrow stromal cells, that is highly relevant to the survival of memory T cells, we could raise a hypothesis on the participation of these cells in T2R genesis. Besides, IL-7 activates the STAT5 transcription factor, which is associated to the expression of anti-apoptotic molecules, such as Bcl-2.[25] In addition, a recent study showed IL-7 activity in enhancing cytokine-producing CD4 pathogen-specific T cells, such as *M. tuberculosis*.[26] Thus, we hypothesize that *M. leprae* antigenic stimulation might increase IL-7 in ENL patients and could be able to induce a synergic effect in cytokine-producing memory T cells.

Therefore, the high levels of this cytokine during T2R may be closely related to the homeostasis of memory cells during this reactional episode. A relevant work using experimental model of infection by herpes simplex virus in the skin of mice revealed the activation of T_{EM} cells, which, even after the withdrawal of the antigenic stimulation, can permanently reside inside peripheral tissues.[27] This fact may be helpful to explain, in part, the occurrence of T2R even on completion of MDT, as well as the incidence of several successive reactional episodes. Nevertheless, one should still clarify which factors lead to the reactivation of these effector memory cells, and, for such, our further objectives encompass performing longitudinal studies with polar lepromatous patients. As arises from our work, the T2R group presented a significantly reduced frequency of IFN-γ–producing T lymphocytes, being such decrease more marked among $CD8^+$ T cells, when compared with the non-reactional LL group. These data were confirmed by the significantly reduced expression of *STAT4* and *TBX21*, transcriptional factors associated with the differentiation for Th1 profile, and characterized by the production of IFN-γ. Among reactional patients, the IL-10–producing naive and central memory T cells, both $CD4^+$ and $CD8^+$, were significantly increased, when compared with the same subsets from non-reactional LL patients. This finding corroborates the thesis on a negative modulation of Th1 profile in triggering T2R. In relation to T2R genesis, one may still possibly suppose that IL-10–producing cells, such as Th2 lymphocytes, show an ability to suppress the differentiation from Th1, as *GATA3* expression inhibits the transcription of the RUNX3 gene, thus reducing the proportion of Th1 cells, as shown by Pham and others.[28] However, the increased frequency of IL-10–producing cells in the immunopathology of T2R requires further investigations. In addition, Treg cells may participate in triggering the reaction, as they present several mechanisms to suppress the immune response, such as the expression of CTLA-4 and ICOS.[29] Nonetheless, we did not observe significant differences between the studied group as to *FOXP3* expression. Thus, the mechanisms of action from Treg in triggering T2R is the focus of our study now.

In addition, there is a relevant interest in assessing the participation of T regulatory type 1 cells (Tr1), namely $CD4^+$ T cells that do not express *FOXP3* but present a regulatory functional activity for being efficient IL-10 producers.[30] In a longitudinal prospective study by using blood leukocytes from AIDS patients, Chevalier et al.[31] demonstrated that Tr1 are the

major IL-10 producers during primary viral infections, even surpassing IL-10 production by Treg. According to the authors, Tr1 cells were proven to play a beneficial role in the immune response control within the context of infection by HIV.

Another key cytokine in the pathogenesis of leprosy reactions is TNF. The serum levels of this pro-inflammatory mediator were found to be increased both in T1R and T2R,[32] not to mention that its mRNA and the protein itself were identified inside the cutaneous lesions.[33,34] The significant increase observed in the frequency of TNF-producing $CD8^+$ T_{EM} among T2R patients led us to correlate these cells with the severity of tissue lesions, as well as to systemic manifestations, particularly high C-Reactive Protein (CRP) levels, leukocytosis, fever, and malaise (data not shown). Of note, acute sepsis-like manifestations were already documented in T2R, including high levels of CRP.[35,36]

We also observed a strong positive correlation between the BI from reactional patients and the frequency of TNF-producing $CD4^+$ T cell in T2R. By contrast, we found a negative correlation between the BI and IL-10^+–producing T cells. Likewise, a study demonstrated that TNF blockade induces IL-10–producing in $CD4^+$ T cells and Th17 cells.[37] Therefore, increased levels of CD4/TNF^+ T cells in ENL may be helpful to reduce the frequency of CD4/IL-10^+ T cells. In view of such data, we hypothesize that the higher the BI from patients during ENL is, the higher will be the levels of pro-inflammatory cytokines, which may result in more severe tissue damages, in addition to physical disabilities for patients. In a recent work, our group demonstrated a positive correlation between BI and the frequency of memory T lymphocytes, and between these cells and the number of lesions in relapsed lepromatous patients.[38] Understanding which alterations in the physiological status of patients are effectively associated with the immune imbalance responsible for triggering T2R remains a challenge.

Anyway, our study discloses the relevant role played by T_{EM} lymphocytes in the immunopathogenesis of T2R, and we conceive that, during reactions, they possibly migrate toward the skin to become activated resident cells, then producing inflammatory mediators that characterize the reactional lesions. This hypothesis should be considered, given the relation between the increased frequency of T_{EM} cells and tissue damage.[39] An interesting recent work from Park and Kupper[40] discussed the role played by these cells in inflammatory diseases. As discussed by the authors, a fundamental feature of memory $CD8^+$ T cells is their ability to rapidly reacquire effector function and massively proliferate on antigen encounter. In this respect, why memory $CD8^+$ T cells are capable of a rapid response to *M. leprae* in T2R is still poorly understood.

Assessing the frequency of this T lymphocyte subset in skin lesions from patients is relevant, as this analysis could demonstrate the migration of these cells from peripheral blood to the tissues. Nevertheless, it was impossible to carry out such a study in this work. So, further investigations about the presence and functional activity of T_{EM} in cutaneous lesions from T2R patients may bring additional elements to the data described herein, thus contributing for a better clarification of molecular and cellular facts associated to the immunopathogenesis of T2R.

Taken together, the results obtained by our group and by other authors suggest using minocycline to T2R prevention and treatment. This is a drug with potential anti-inflammatory effect, anti-apoptotic properties, and inhibitor of angiogenesis. In addition, minocycline presents a neuroprotector effect, and its use can prevent both the onset of the reactional episode and neuropathic pain provoked by acute inflammation.[41–43] Robust clinical studies are still required to validate the use of this drug for such purpose.

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