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From Cell Biology to Tissue Engineering

Immunohistochemical assessment of cell populations in leprosy-spectrum lesions and reactional forms

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Summary. *In situ* immunophenotyping of leprosy lesions can improve our understanding of the biology of inflammatory cells during the immune response to *Mycobacterium leprae* antigens. In the present study, biopsies from 10 healthy controls and 70 leprosy patients were selected, 10 for each of the following conditions: clinical tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), lepromatous (LL), reversal reaction (R1), and erythema nodosum leprosum (R2). Qualitative and quantitative immunohistochemical analyses were performed to detect CD3, CD4, CD8, FoxP3, CD20, CD138, CD1a, CD57, CD15, CD117, CD68, and CD163. In addition, histochemistry was employed to identify eosinophils. The amount of $CD3^+$ and $CD4^+$ T cells was higher in TT than in LL patients. $CD8^+$ T cells were predominant in T lymphocyte infiltrations in the basal layer of the epidermis. The number of $FoxP3^+$ cells was similar among different forms of the disease, but was higher in BL and LL than in R2 individuals. $CD20^+$ lymphocytes were most abundant in TT samples, while $CD138^+$ plasma cells displayed no detectable differences. Epithelioid macrophages from the center of TT and R1 granulomas exhibited the M1 phenotype ($CD68^+CD163^-$), whereas those in LL granulomas showed the M2 phenotype ($CD68^+CD163^+$). There was a gradual decrease in the amount of $CD1a^+$ cells from the TT towards the LL form of the disease. A significant increase in the number of neutrophils was observed only in R2 samples. All the cells investigated, except eosinophils, participated in the immunopathogenesis of leprosy.

Key words: Leprosy, *M. leprae,* Pathogenesis, Cellular infiltrate, Immunopathology

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Introduction

Leprosy is a chronic disease caused by *Mycobacterium leprae*, an obligate intracellular bacillus with tropism for the peripheral nervous system. The host's cell-mediated immune response determines the pattern and progression of the disease within a spectrum encompassing different histopathological, bacilloscopic, and clinical characteristics. At one extreme, the tuberculoid (TT) form of leprosy is characterized by a high level of host resistance and well-organized granulomas composed of epithelioid macrophages, lymphocytes, and few or no bacilli. At the other end, lepromatous leprosy (LL) exhibits low-level host resistance and extensive granulomas composed of multi-vacuolated macrophages, with few lymphocytes and abundant bacilli. Lepromatous forms include histoid leprosy, a special form of leprosy associated with drug resistance to dapsone. The forms of leprosy that fall between these two extremes are known as borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL). The borderline forms involve partial immunity against *M. leprae*. This unstable immunity enables evolution of the immune response towards tuberculoid (upgrading) or

lepromatous (downgrading) forms (Ridley and Jopling, 1966; Hastings et al., 1988; Fleury, 2000). Some patients experience acute and frequent episodes of inflammation known as reactions. There are two types of reactions in leprosy. Type "1" reaction (R1) or Reversal Reaction (RR) occurs in patients for whom specific cellular immunity against *M. leprae* is preserved to some degree (TT, BT, BB, and BL). Type "2" reaction (R2) or erythema nodosum leprosum (ENL) occurs in patients displaying barely preserved or virtually absent cellular immunity (BL and LL) (Soares et al., 2013). Both types of reactions can cause permanent neurological complications (Ridley and Jopling, 1966; Hastings et al., 1988; Fleury, 2000).

The inflammatory process associated with leprosy can progress over years or sometimes decades. During this time, the continuous recruitment of various cell populations at different stages of maturation leads to granulomas of various disease and reactional forms. Therefore, the cell-mediated immune response towards *M. leprae* antigens results in the complex cellular composition of leprosy lesions. In the present study, we sought to apply our knowledge of immune cells to the pathophysiology of leprosy.

Materials and methods

Case selection

Selected biopsy specimens from the archives of the Laboratory of Anatomical Pathology, Lauro Souza de Lima Institute (ILSL), Bauru, São Paulo, Brazil, were analyzed retrospectively. Seventy blocks containing punch biopsies of skin lesions from individuals with leprosy collected between January 2007 and December 2010 were selected. Ten blocks containing skin biopsies lacking histopathological changes were used as controls. The inclusion criteria were: clinical diagnosis of leprosy confirmed by dermatoneurological diagnosis, Ridley and Jopling's classification (Ridley and Jopling, 1966), in different disease forms, and naïve leprosy treatment. Reactional patients were treated with multidrug therapy but not with corticosteroid or thalidomide. The samples included: 10 controls and 10 specimens per each leprosy and reactional form (n=70) (Fig. 1). The study was approved by the research ethics committees of the ILSL (CEP 021/2011) and the Federal University of São Paulo/São Paulo Hospital (CEP 0954/11).

Immunohistochemical (IHC) and histochemical (HC) techniques

IHC analysis was performed indirectly using an ADVANCED® HRP kit (Dako, Carpinteria, CA, USA), as described previously (Taylor et al., 2010). The antibodies used to detect different types of cells, the dilutions, and the associated antigen-retrieval methods are described in Table 1. Histochemical staining was performed using Giemsa's azur eosin methylene blue solution (Merck KGaA, Darmstadt, Germany).

Quantitative analysis

Nine fields corresponding to the areas with the greatest clustering of immunostained cells (spots) were analyzed per slide using a low (10×) magnification on an Axiophot 2 microscope (Zeiss, Gottingen, Germany). These consisted of three fields each of papillary dermis/epidermis, the middle portion of the dermis, and the deep layer of the dermis/subcutaneous tissue. One high-magnification image (40×) was acquired per spot and a grid with an area of approximately 243 μm^2 was applied (AxioCam, Zeiss) (Fig. 1E). The acquired images were analyzed using Axion 4.7 software (Zeiss). The morphological and immunostaining characteristics of each cell were assessed by two examiners (L.R.V.F. and C.T.S.) and differences were resolved by consensus. Only immunostained cells with nuclei located within the

Table 1. List of the antibodies used to identify cell populations.

Cell	Antibody primary	Clone	Mark	Dilution	Antigen retrieval
T lymphocyte	CD3	SP7	NEOMARKERS	1/1000	Sodium citrate
Helper T lymphocyte	CD4	4B12	DAKO	1/100	Sodium citrate
Cytotoxic T lymphocyte	CD8	C8/144B	DAKO	1/200	Sodium citrate
T regulatory cell	FoxP3	SP97	SPRING BIOSCIENCE	1/200	Tris-EDTA pH 9.0
Natural killer cell	CD57	NK-1	NOVOCASTRA	1/100	PBS
B lymphocyte	CD20cy	L26	DAKO	1/1000	Sodium citrate
Plasm cell	CD138	MI15	DAKO	1/400	Sodium citrate
Cells of monocyte/macrophage lineage in normal and pathologic conditions	CD68	PG-M1	DAKO	1/400	Sodium citrate
Restricted to monocytes and tissue macrophages	CD163	10D6	NOVOCASTRA	1/400	Sodium citrate
Antigen presenting cell	CD1a	O10	DAKO	1/200	Tris-EDTA pH 9.0
Neutrophil	CD15	C3D-1	DAKO	1/400	Sodium citrate
Mast cell	CD117	YR145	DAKO	1/200	Sodium citrate

NeoMarkers, Fremont, CA, USA; Dako, Carpinteria, CA, USA; Spring Bioscience, Pleasanton, CA, USA; Novocastra, Newcastle, UK; Tris= tris(hydroxymethyl)-aminomethane; EDTA: ethylenediaminetetraacetic acid; pH: hydrogen ion concentration; PBS: phosphate-buffered saline.

grid intersection were counted (Fig. 1E). The number of labeled cells per slide corresponded to the mean number of cells in the evaluated fields; the latter was calculated as the mean value of 10 slides with the same labeled marker. CD4:CD8 and CD68:CD163 ratios were calculated by dividing the mean number of cells per field labeled with one marker by the mean number of cells per field labeled with another marker.

Qualitative analysis

Cell distribution in the samples was assessed jointly by two examiners (L.R.V.F. and C.T.S.). Analyzed features included epidermis, appendages (hair follicles and glands), arrector pili muscle, interstitium, blood vessels, nerve branches, and granulomas (Fig. 1F). Immunostained cells (IHC or HC) and their relative

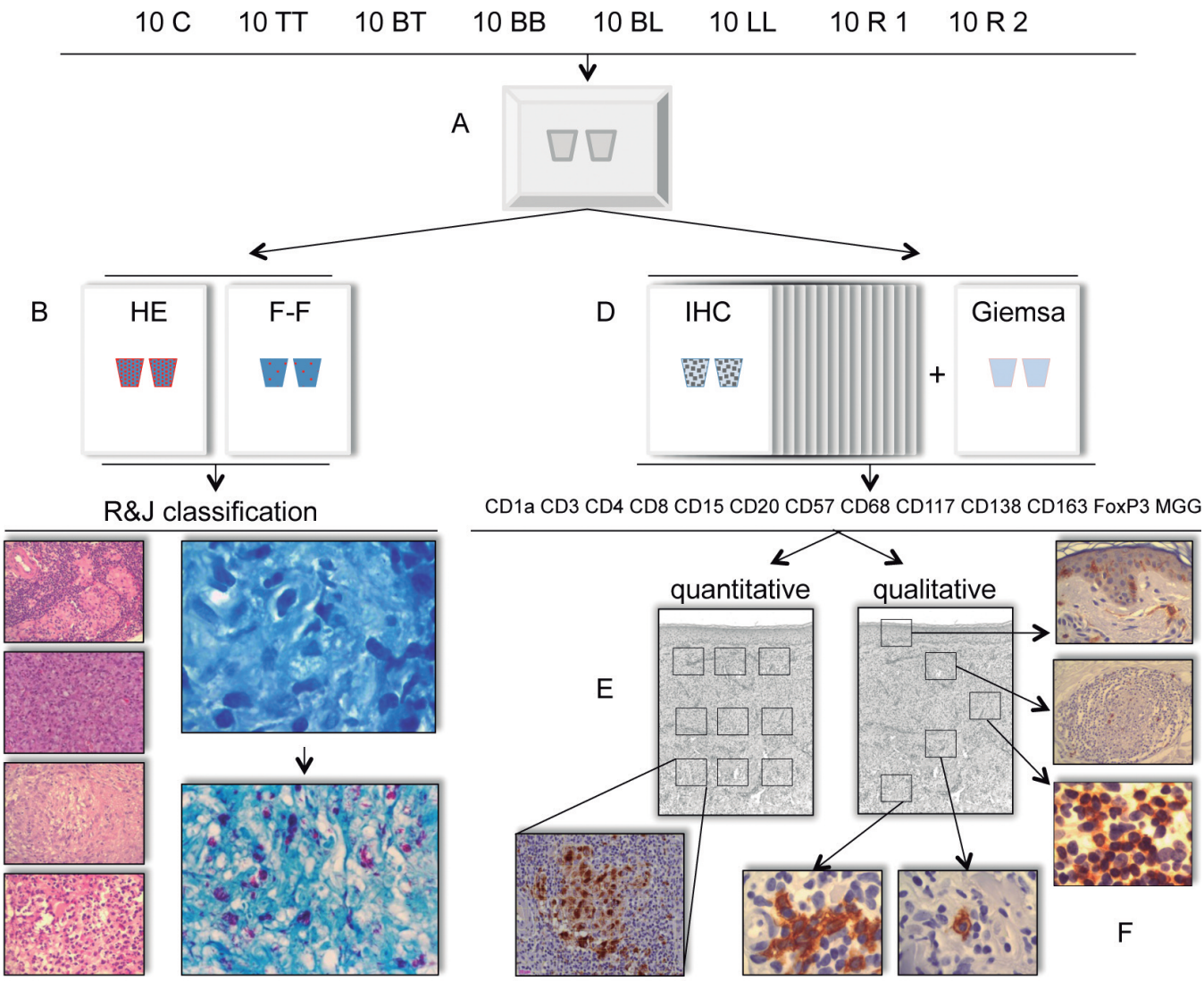

Fig. 1. Study design. **A.** Selected blocks of punch biopsies from cutaneous lesions of patients with various forms of leprosy (TT, BT, BB, BL, and LL), type of reaction (R1 and R2), and skin samples from healthy controls (C). **B.** Tissue sections stained with hematoxylin-eosin (H&E) and the Faraco-Fite (F-F) procedure (to stain bacilli). **C.** Reaction characterization according to the Ridley-Jopling (R&J) classification. **D.** Immunohistochemical characterization of cell populations using histochemistry (Giemsa) and antibodies against CD1a, CD3, CD4, CD8, CD15, CD20, CD57, CD68, CD117, CD138, CD163, and FoxP3. **E.** Quantitative assessment of each marker by means of cell counts in nine high-magnification (243 μm^2) fields (spots). **F.** Qualitative assessment of cell distribution in skin components (epidermis, skin appendages, blood vessels, nerves, interstitium, and granulomas). TT, tuberculoid (n=10); BT, borderline tuberculoid (n=10); BB, borderline borderline (n=10); BL, borderline lepromatous (n=10); LL, lepromatous (n=10); R1, type 1 reaction (n=10); and R2, type 2 reaction (n=10).

predominance were assessed for each component in decreasing order: granuloma > nerve > vessel > interstitium > skin appendages > epidermis.

Statistical analysis

The groups were compared using non-parametric models. The results are presented as the mean number of cells per field. The Mann-Whitney U test was used to evaluate significant differences in pairwise comparisons. Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Prism Software Inc., La Jolla, CA, USA) at a $p<0.05$ significance level. The following comparisons were performed for each marker: leprosy patients (TT + BT + BB + BL + LL + R1 + R2) vs. controls, extreme forms (TT vs. LL), tuberculoid side (TT side: TT+BT) vs. lepromatous side (LL side: BB + BL + LL), R1 individuals vs. individuals susceptible to R1 (R1-S: TT + BT + BB + BL), and R2 individuals vs. individuals susceptible to R2 (R2-S: BL + LL).

Results

CD3⁺, CD4⁺, CD8⁺, FoxP3⁺ T lymphocytes, and CD57⁺ natural killer (NK) cells

The number of $CD3^+$ T lymphocytes was significantly higher in leprosy patient vs. control, in TT vs. LL, in TT side vs. LL side, and in R1 vs. R1-S specimens (Fig. 2A).

Histological analysis showed that in control samples some $CD3^+$ cells were distributed around the capillaries in the papillary dermis. Instead, in TT, BT, and R1 samples, $CD3^+$ cells predominated in the periphery of granulomas and were rarely observed in the center. In BB, BL, and LL samples they were present around and inside the granulomas. In R2 biopsies, $CD3^+$ T cells appeared in the periphery of pre-existing granulomas and were scarce or absent in micro-abscesses. The distribution pattern of this cell population was: granuloma > perivascular > perineural > periadnexal > interstitium > epidermis (data not shown).

The amount of $CD4^+$ T cells was significantly higher in patient vs. control, in TT vs. LL, TT side vs. LL side, and in R2 vs. R2-S samples (Fig. 2B). Histological analysis revealed that the few $CD4^+$ T cells found in controls were distributed around the capillaries of the papillary dermis. In TT and BT samples, $CD4^+$ T cells predominated in the periphery of granulomas and exhibited higher organization in the deep layer of the dermis; whereas in BB, BL, LL, and R1 biopsies, these cells were present around and within granulomas. Finally, in R2, $CD4^+$ T cells were observed in pre-existing granulomas, but were sparse or absent in micro-abscesses (data not shown).

The number of $CD8^+$ T cells was significantly higher in patient vs. control and in R2-S vs. R2 samples (Fig. 2C). The amount of $CD4^+$ and $CD8^+$ cells was

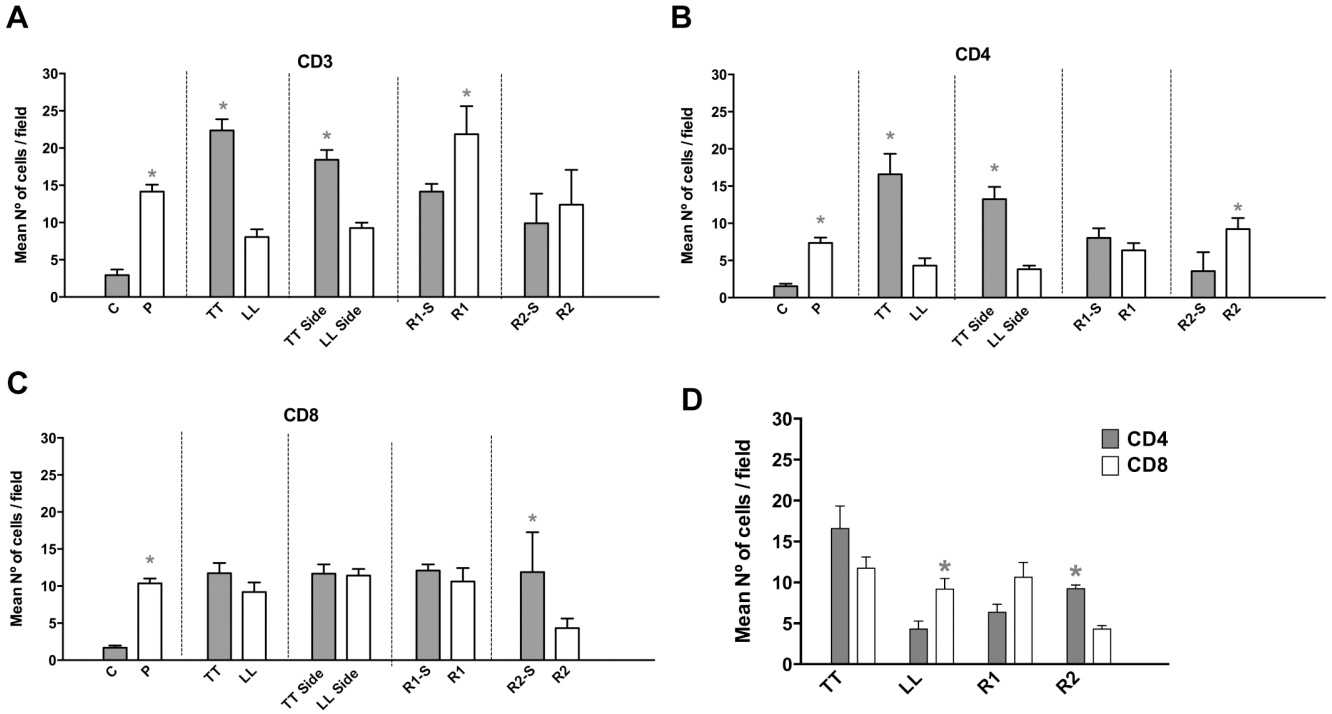

Fig. 2. Quantitative analysis of CD3+ **(A)**, CD4+ **(B)**, and CD8+ cells **(C)** in leprosy skin biopsies. **D.** CD4:CD8 ratio. *p<0.05, Mann-Whitney U-test.

similar in TT samples, whereas $CD8^+$ cells were more abundant than $CD4^+$ cells in LL specimens (Fig. 2D). The CD4:CD8 ratio was inverted in TT and LL samples, with values of 1.4:1 and 1:2.1, respectively. The CD4:CD8 ratio was 1:1.6 in R1 and 2.1:1 in R2 specimens (Fig. 2D). The distribution pattern of $CD8^+$ cells among the various forms of the disease and reaction types was similar to that of $CD4^+$ and $CD3^+$ cells. However, two singular aspects were observed. First, among the lymphocytes that intermingled with squamous cells and melanocytes in the epidermis, $CD8^+$ cells (Fig. 3A) were clearly more abundant than $CD4^+$ cells (Fig. 3D). Second, the number of lymphocytes in the epidermis was lower in BB and LL than in TT samples (Fig. 3B,C,E,F).

The amount of $FoxP3^+$ lymphocytes was significantly higher in patient vs. control and in R2-S vs. R2 samples (Fig. 4A). The distribution pattern of $FoxP3^+$ cells was similar across the different disease forms and reactions. These cells were present within granulomas, especially those of the papillary dermis, and were randomly arranged in the lesions. The number of $FoxP3^+$ lymphocytes was lower in the lesions of individuals with borderline forms of leprosy (data not shown).

The number of NK $CD57^+$ lymphocytes was

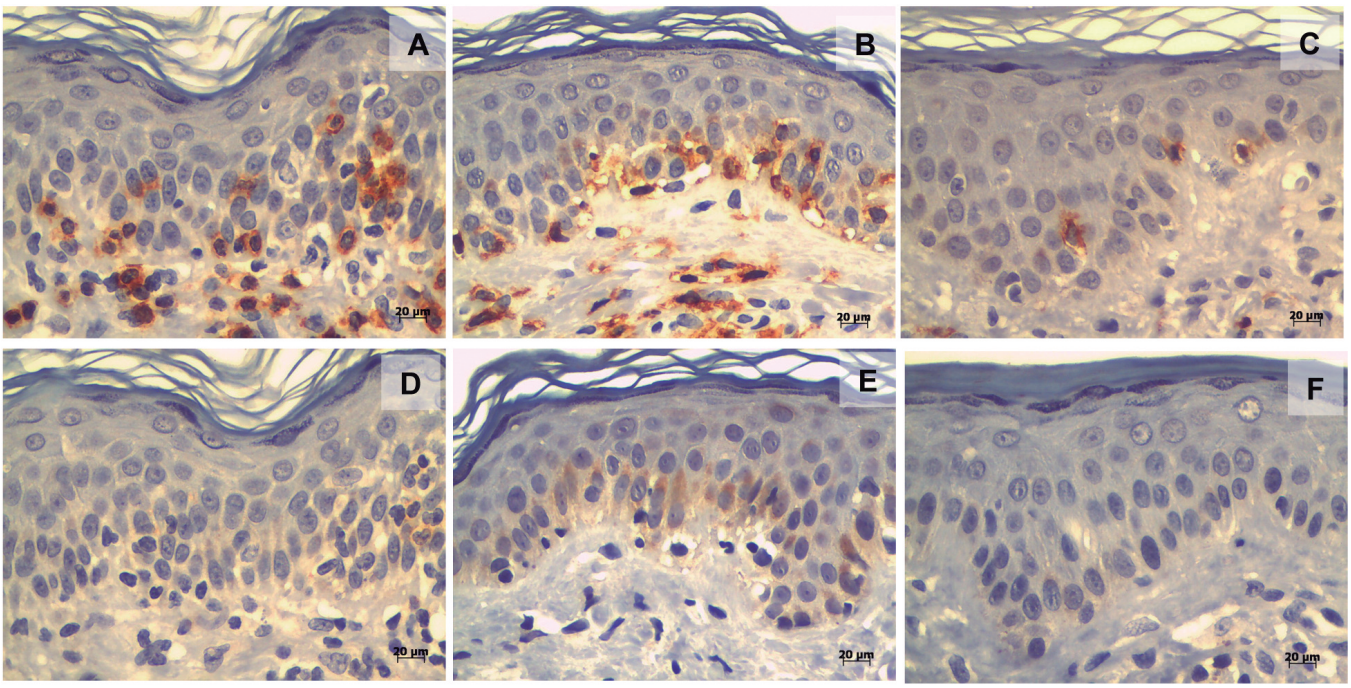

Fig. 3. Expression of CD4+ and CD8+ T lymphocytes in serial sections of skin biopsies. CD8+ T lymphocytes in the epidermis and papillary dermis of TT **(A)**, BB **(B)**, and LL lesions **(C)**. Few CD4+ T lymphocytes can be observed in the epidermis and papillary dermis of TT **(D)**, BB **(E)**, and LL lesions **(F)**. The number of CD4+ and CD8+ T lymphocytes in lesions is seen to decrease from the TT to the LL end of the disease. × 40

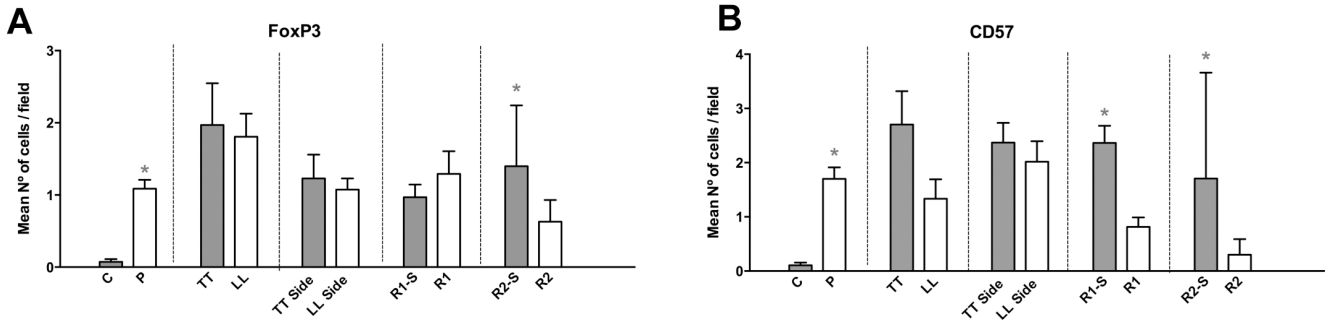

Fig. 4. Quantitative analysis of FoxP3+ **(A)** and CD57+ cells **(B)** in leprosy skin biopsies. *p<0.05, Mann-Whitney U-test.

significantly higher in patient vs. control, R1-S vs. R1, and R2-S vs. R2 samples (Fig. 4B). In the various disease forms and reaction types, lymphocytes were localized predominantly in the periphery of granulomas and were rarely observed inside. In R2 samples, NK CD57+ lymphocytes were seldom observed in the pre-existing granulomas and were totally absent from micro-abscesses. The cell distribution pattern was: granuloma > vascular > perineural > periadnexal > interstitium > epidermis (data not shown).

B lymphocytes (CD20+) and plasma cells (CD138+)

The number of CD20+ B lymphocytes was significantly higher in patient vs. control, TT vs. LL, and TT side vs. LL side samples (Fig. 5A). These cells were entirely absent or very rarely found near the capillaries of control samples. In TT and BT biopsies they formed dense clusters in the periphery of granulomas, with some being observed also in the center (Fig. 5C). In LL side specimens, they were seen in both the periphery and within granulomas (Fig. 5D). CD20+ B lymphocytes were absent from the epidermis and were localized predominantly near the deeper granulomas in reactional samples, while being scarce or absent in micro-abscesses in R2 biopsies. The cell distribution pattern was: granuloma > perivascular > perineural > periadnexal > interstitium (data not shown).

The number of CD138+ plasma cells was significantly higher in patient vs. control samples (Fig. 5B); no other differences were observed. In TT, BT, and R1, these cells were localized predominantly in the periphery of granulomas and occasionally formed small clusters. In LL side specimens, they were found in the periphery and center of granulomas, as well as in perivascular spaces. In R2 samples, CD138+ plasma cells were localized in the periphery of pre-existing granulomas and near blood vessels, but were absent from micro-abscesses (data not shown).

CD68+ and CD163+ macrophages

The number of CD68+ or CD163+ macrophages was significantly higher in patient vs. control, LL vs. TT, and R1 vs. R1-S samples (Fig. 6A,B). In addition, CD163+ macrophages were significantly more abundant also in LL side vs. TT side and R2-S vs. R2 samples (Fig. 6B). In TT side specimens, CD68+ macrophages were predominantly located in the center of granulomas, with a smaller number situated in the periphery (Fig. 6C). Few such cells were observed near blood vessels, skin appendages, and in the interstitium. In LL side samples, these macrophages were located both within and at the periphery of the granulomas (Fig. 6D). In R1 samples,

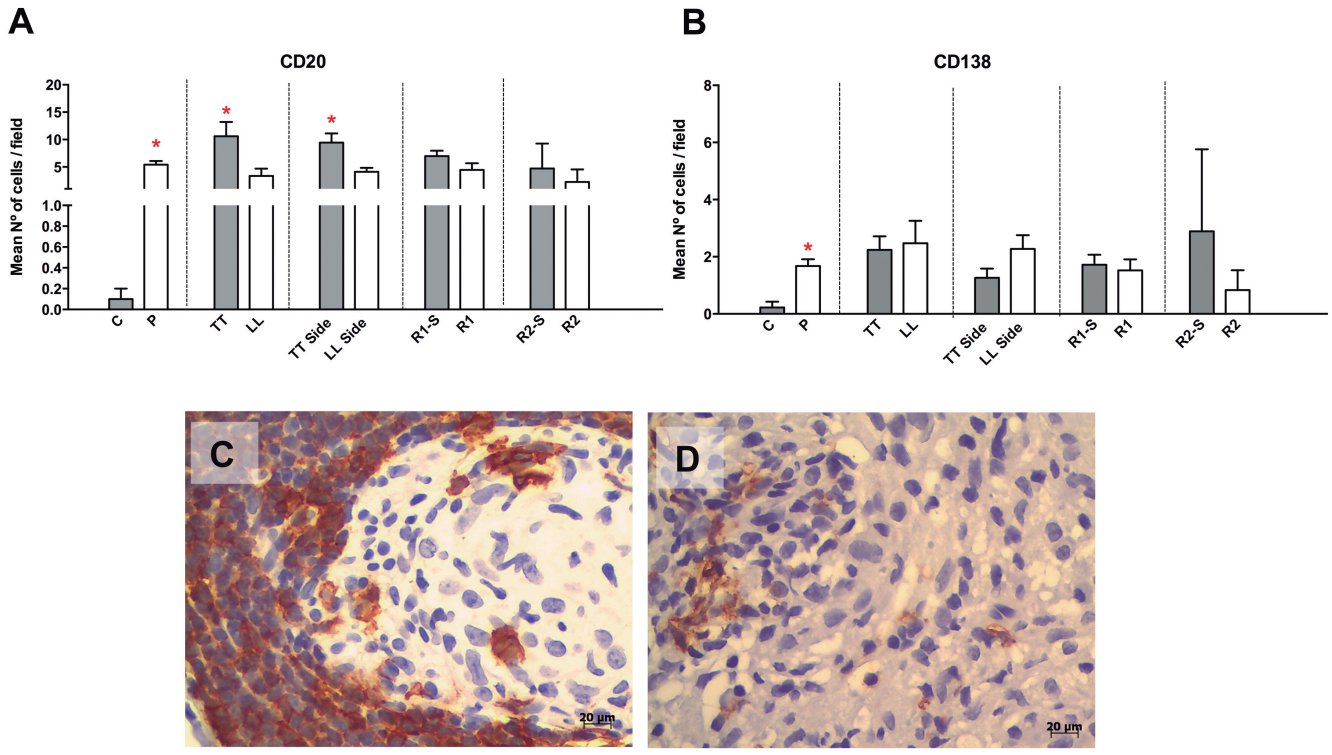

Fig. 5. Quantitative analysis of CD20+ **(A)** and CD138+ cells **(B)** in leprosy skin biopsies. *p<0.05, Mann-Whitney U-test. **C.** Image showing abundant CD20+ B lymphocytes in the periphery of a tuberculoid granuloma. **D.** Image depicting CD20+ B lymphocytes in a lepromatous granuloma. × 40

they were observed throughout granulomas exhibiting reactions (Fig. 6E) and in R2 specimens they were present in pre-existing lesions as well as in the periphery of micro-abscesses (Fig. 6F). The cell distribution pattern was granuloma > perineural> perivascular > interstitium > periadnexal.

Qualitative analysis indicated that the epithelioid macrophages at the center of granulomas in TT samples (Fig. 6G), and the new macrophages and multinucleated giant cells in granulomas of R1 biopsies, corresponded to $CD163^-$ cells (Fig. 6I). $CD163^+$ macrophages were less abundant in the periphery of TT granulomas (Fig. 6G) and R1 samples (Fig. 6I), but were found throughout granulomas in LL samples (Fig. 6H). In R2 samples, $CD163^+$ macrophages were found in pre-existing granulomas, but were absent from micro-abscesses (Fig. 6J). BT results were comparable to those from TT specimens, while BB and BL were similar to LL samples.

Qualitative analysis of the distribution of $CD68^+$ and $CD163^+$ macrophages revealed that the expression of M1 and M2 macrophages was clearly defined in TT side and R1 samples. In TT side biopsies, the epithelioid macrophages located in the center of granulomas exhibited almost exclusively the M1 phenotype ($CD68^+CD163^-$) (Fig. 6C,G), as did also young macrophages that had infiltrated and contributed to the reactive phenomenon in pre-existing R1 granulomas (Fig. 6E,I). The M2 phenotype ($CD68^+CD163^+$) was absolute in LL macrophages (Fig. 6D,H) and predominant in R2 macrophages that were part of pre-existing granulomas. In addition, a small number of M1 macrophages were observed near neutrophils in micro-abscesses (Fig. 6F,J).

Neutrophils ($CD15^+$), dendritic cells ($CD1a^+$), mast cells ($CD117^+$), and eosinophils (Giemsa)

The number of neutrophils was significantly higher in patient vs. control and R2 vs. R2-S samples (Fig. 7A). Neutrophils were seldom observed in the capillaries of control and R1 samples and in various forms of the

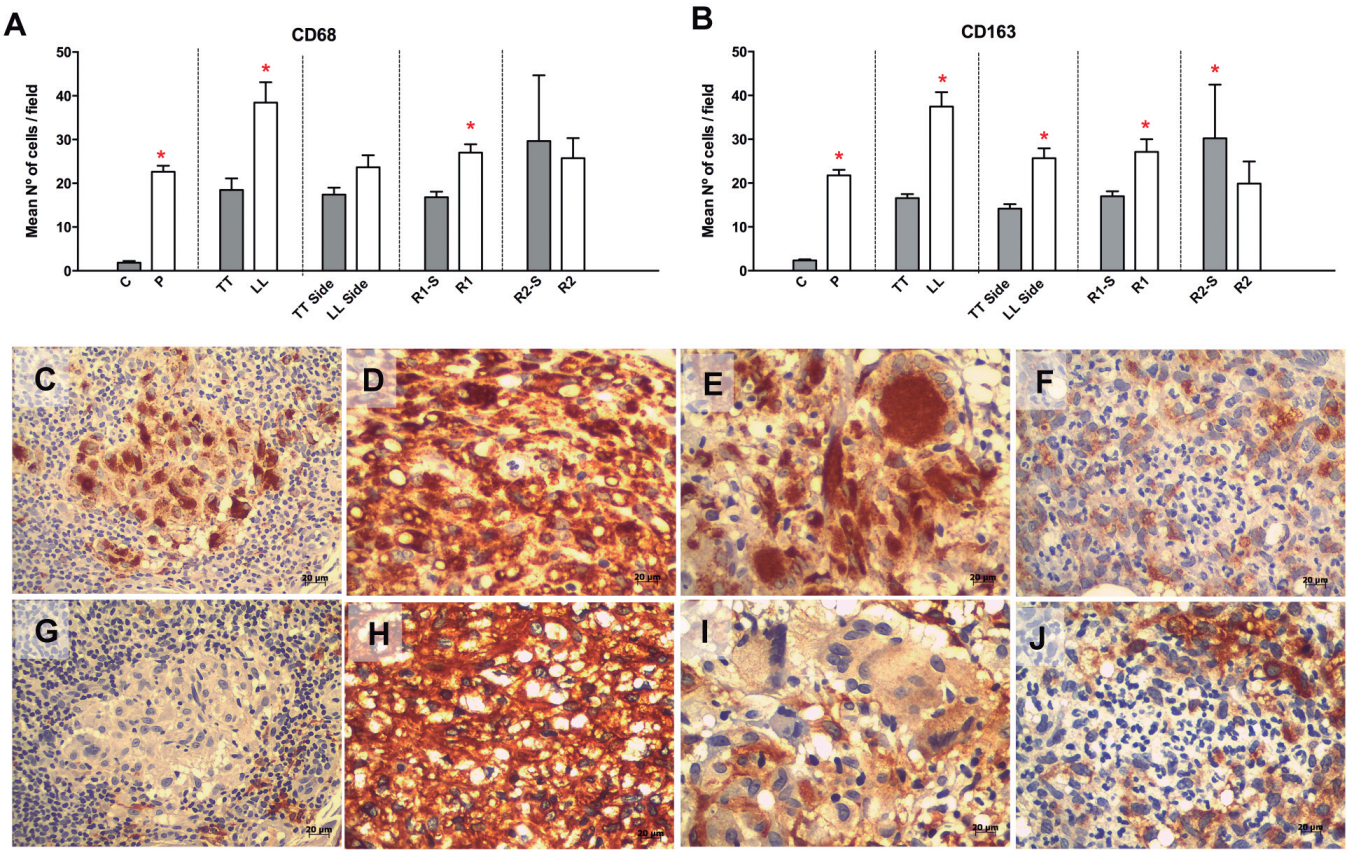

Fig. 6. Quantitative analysis of CD68+ **(A)** and CD163+ **(B)** cells in leprosy skin biopsies. *p<0.05, Mann-Whitney U-test. Images of a tuberculoid granuloma showing CD68+ macrophages **(C)** localized primarily in the center and a few peripheral CD163+ macrophages **(G)**. Images of full-length lepromatous granuloma displaying CD68+ **(D)** and CD163+ macrophages **(H)**. Images of R1 lesions showing CD68+ macrophages **(E)** in the center and periphery and predominantly peripheral CD163+ macrophages **(I)**. R2 lesions showing the peripheral distribution of CD68+ **(F)** and CD163+ macrophages **(J)**. C, G, × 20; D-F, H-J, × 40

disease. In R2 specimens, a large number of neutrophils were observed predominantly in micro-abscesses and on the walls of, and within, the lumen of blood vessels. Few neutrophils were found within pre-existing granulomas and hardly any in the epidermis (data not shown).

The number of CD1a$^+$ cells was significantly higher in control vs. patient, TT vs. LL, and TT side vs. LL side samples (Fig. 7B). The amount of CD1a$^+$ cells decreased gradually from TT to the borderline and LL lesions. In all cases, CD1a$^+$ cells were distributed throughout the epidermis, near skin appendages, and surrounding blood vessels, while their occurrence in granulomas was uncommon (data not shown).

The number of CD117$^+$ mast cells was significantly higher in TT vs. LL and TT side vs. LL side samples (Fig. 7C). Mast cells were localized predominantly in the perivascular, periadnexal, and perineural areas of the superficial region of the dermis. In R2 specimens, no mast cells were observed in micro-abscesses (data not shown).

The amount of eosinophils was negligible in the various disease forms and reaction types; they were also absent from control samples. Eosinophils were not observed in lesions and only rarely in capillaries (data not shown).

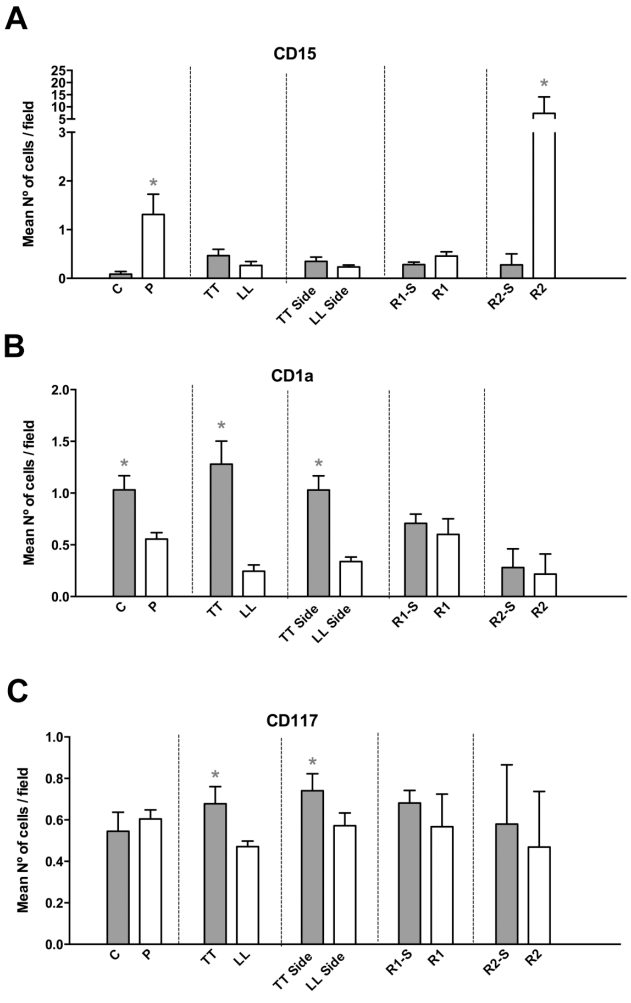

Fig. 7. Quantitative analysis of CD15+ **(A)**, CD1a+ **(B)**, and CD117+ cells **(C)** in leprosy skin biopsies. *p<0.05, Mann-Whitney U-test.

Discussion

In leprosy, the immune response is mediated by CD4 T cells, macrophages, and pro-inflammatory cytokines such as tumor necrosis factor (TNF) α (Oliveira et al., 2003; Rodrigues and Lockwood, 2011). In line with previous studies, we observed higher numbers of CD3$^+$ T lymphocytes in TT and BT samples. Assessment of the CD4:CD8 ratio provides a measure of the relative amounts of helper:suppressor T cells. Narayanan et al. (1983) reported CD4:CD8 ratios of 1:1, 1.7:1, and 0.4:1 for TT, BT, and LL lesions, respectively. Modlin et al. (1988) observed that the CD4:CD8 ratio was 1.7:1 and 0.6:1 in TT and LL lesions, respectively. Our results agree in part with those reported in the literature, namely, a CD4:CD8 ratio of 1.4:1 in TT lesions and 1:2.1 in LL lesions. The content of CD4$^+$ and CD8$^+$ cells was similar in all skin components except among T lymphocytes that had infiltrated the basal layer of the epidermis, where CD8$^+$ were more abundant than CD4$^+$ cells. The hypochromic appearance of leprosy lesions may be the result of CD8$^+$ cells attacking melanocytes and keratinocytes in the basal epidermal layer. Epidermotropic CD8$^+$ cells have been described as the cause of hypopigmentation in other diseases, such as mycosis fungoides and vitiligo (Furlan and Sanches, 2013; Yang et al., 2015).

T regulatory cells (Tregs) have been hypothesized to facilitate the persistence of *M. leprae* in lepromatous patients (Palermo et al., 2012; Bobosha et al., 2014; Saini et al., 2014). Our results agree with previous reports, whereby this population of lymphocytes was observed in all patients with leprosy, independently of the disease form (Massone et al., 2010; Parente et al., 2015). Tregs most likely play a variety of roles depending on the context, as in the case of particular pathogens or at different stages of infection. In the case of TT lesions, which are characterized by an inflammatory T helper 1 (Th1)-rich environment, FoxP3$^+$ cells may act to restore homeostasis following bacillary clearance. Furthermore, Tregs play beneficial roles such as blocking the apoptotic cascade. Given the correlation between apoptotic activity and Tregs, a moderate synergistic effect between caspase-3 and FoxP3 was reported in the tuberculoid form of the disease (Quaresma et al., 2014). In LL lesions, which are characterized by an anti-inflammatory Th2-rich environment, these cells might contribute to immunosuppression of the Th1 response, thus allowing the persistence and proliferation of bacilli.

Reactional episodes in leprosy are result from

complex interactions between the immune system and *M. leprae*. We observed a distinct behavior among patients of R1 and R2 reaction types. Whereas the former showed an increase in the number of Tregs in lesions compared to R1-S patients, the latter displayed a significant decrease of Tregs compared to R2-S individuals. This suggests a failure in the immune regulation of the inflammatory process, leading to hyperactivation of the immune response, which is a common feature of the R2 reactional episode. This phenomenon was well characterized by Modlin et al. (1988) and is confirmed here by a high CD4:CD8 ratio in R2 lesions. It is possible that in such patients part of these $CD4^+$ T lymphocytes have initiated a Th17 response (Attia et al., 2014; Quaresma et al., 2015) rather than an immunosuppressive response, thus justifying the intense neutrophilic infiltrate observed in R2 lesions. Furthermore, the association between interleukin (IL)-6 and the occurrence of R1 and R2 has been reported earlier (Teles et al., 2002; Stefani et al., 2009). IL-6 is a multifunctional cytokine with a key role in the acute immune inflammatory response. It determines the balance between Treg ($FoxP3^+$) and Th17 lymphocytes, suppresses the generation of Treg transforming growth factor (TGF)-β, and leads to Th17 differentiation (Kimura et al., 2007). It is also evident that $CD4^+$ T cells can be differentiated into Th22. Higher levels of IL-13 and IL-22 associate with the formation of immune complexes and the development of a Th22 response in R2 susceptible leprosy (Silveira et al., 2015).

R1 reactions are characterized by an increase in the cellular immune response with activation of macrophages and production of Th1 cytokines, such as interferon (IFN)-γ and IL-12 (Little et al., 2001). Considering that Tregs were observed to increase in R1 compared to R1-S samples, they have been suggested to suppress the exacerbated inflammatory response of R1 episodes.

The number of NK $CD57^+$ cells was similar among various disease forms and reaction types. Belone (2004) observed that the density of NK cells *in situ* was higher in TT and BT lesions than in R1. Most likely, NK cells remain in the leprosy lesions following the innate response and participate in immunopathogenesis of the disease, independently of its clinical form, without significant direct involvement in the reactions.

In contrast to previous reports (Ridley, 1974), we observed a greater number of $CD20^+$ B lymphocytes in tuberculoid than in lepromatous leprosy. Depending on the signals that B lymphocytes receive when presenting antigens to naive CD4 T cells, the former may produce pro-inflammatory or anti-inflammatory cytokines (Hamze et al., 2013; Vadasz et al., 2013). In tuberculoid leprosy, B lymphocytes might contribute to the effector stage of the immune response by producing cytokines, such as IL-2, IL-12, IL-4, and IFN-γ (Harris et al., 2000), or in the maintenance of tolerance via the expression of regulatory cytokines, such as IL-10 and TGF-β (Mauri and Bosma, 2012). The role of the B-lymphocyte population in down-regulation of the immune response in lepromatous leprosy is unknown.

Antibodies secreted by plasma cells in the bone marrow and lymph nodes might compensate for a small population of *in situ* plasma cells. However, the genes associated with B-lymphocyte functions were found to be more expressed in LL and R2 than in TT samples, and plasma cells were more abundant in LL than in TT lesions (Ochoa et al., 2010).

M1 macrophages produce pro-inflammatory cytokines (IFN-γ, IL-12, TNF-α, IL-6, IL-1β, and IL-23), chemokines (CCL2, CXCL10, and CXCL11), co-stimulatory molecules, major histocompatibility complex (MHC) molecules (Tomioka et al., 2012; Martinez and Gordon, 2014), and reactive nitrogen and oxygen intermediates. They also promote the Th1 response, and exhibit strong microbicidal and tumoricidal activities (Sica et al., 2008). M2 macrophages express the scavenger receptor CD163, secrete IL-10 and TGF-β, have an immunomodulatory effect, and exhibit poor microbicidal activity (Tomioka et al., 2012).

With respect to the macrophage population, in the present study the epithelioid macrophages at the centre of tuberculoid granulomas exhibited the M1 phenotype, whereas M2 macrophages were localized at the periphery. The spatial organization of macrophage subtypes in tuberculoid granulomas appears to follow a specific microenvironment for microbicidal and homeostatic functions. In tuberculoid granulomas, the central area is probably pro-inflammatory, consisting of M1 macrophages tasked with containing the proliferation of bacilli, as suggested by the presence of very few such microbes. Instead, the peripheral area is anti-inflammatory with predominance of M2 macrophages involved in limiting the tissue damage promoted by the antimicrobial activity of M1 macrophages. In lepromatous, BB, and BL granulomas virtually all macrophages are of the M2 phenotype.

Several *in vivo* studies have indicated that the phenotype of a given population of macrophages might change over time in response to stimulation by different cytokines (Buechler et al., 2000; Tiemessen et al., 2007; Mosser and Edwards, 2008; Murray and Wynn, 2011; Mantovani et al., 2013; Martinez and Gordon, 2014). Lepromatous leprosy is characterized by IL-10 over-expression, which might amplify M2 polarization (Palermo et al., 2012; Saini et al., 2014; de Souza et al., 2016). The expression profile of those cells revealed that mRNA levels of genes associated with the M2 phenotype were greater in LL than in TT samples (Montoya et al., 2009). The *M. leprae*-induced expression of CD163 might modulate the macrophage phenotype, resulting in a favorable environment for bacterial survival (Lee et al., 2010).

In this study we demonstrated for the first time the distribution of M1 and M2 macrophages in leprosy reactional skin lesions. In R1 lesions, the young macrophages that had infiltrated the pre-existing

granulomas and sometimes formed giant cells were predominantly of the M1 phenotype. This phenomenon most likely resulted in bacillary fragmentation and a reduced bacilloscopic index in R1 lesions. This is consistent with increased nitric oxide synthase (iNOS) and TGF-β in such lesions (Lockwood et al., 2011). Our results show that relative to R1 lesions, R2 lesions contained fewer M2 macrophages, although many M1 macrophages were observed near R2 micro-abscesses. These findings indicate that the acute R2 stage is not associated with recruitment of considerable numbers of macrophages, but rather that these are part of pre-existing BL and LL granulomas. The influx of neutrophils and bacillary fragmentation in micro-abscesses suggests that M1 macrophages play a role in microbe clearance and tissue repair mechanisms.

Large numbers of neutrophils were observed only in R2 samples, in agreement with previous reports (Fleury, 2000). The mechanisms by which neutrophils are attracted to leprosy lesions and which determine the occurrence of the R2 reaction are unknown. Changes in the microenvironment of lepromatous lesions during the course of the disease and after chemotherapy might induce extensive bacillary fragmentation and contribute to the influx of neutrophils into the lesions. However, bacillary fragmentation also occurs in other forms of the disease and in lesions of the R1 type in the absence of neutrophil infiltration.

As described in the literature (Sieling et al., 1999; Miranda et al., 2007), we observed that $CD1a^+$ cells were more abundant in TT than in LL samples. *M. leprae* infection of dendritic cells leads to a decrease in the levels of class I and II MHC molecules (Hashimoto et al., 2002), resulting in less competent T cells. $CD1a^+$ cells may increase the effectiveness of the cell-mediated immune response in tuberculoid forms of the disease, because few of these cells were observed in non-tuberculoid forms in which this immune response was less effective.

As in the present study, other authors have observed a greater number of mast cells in tuberculoid (Cree et al., 1990; Myrosekar et al., 2001; Magalhães et al., 2008) and lepromatous patients (Bagwan et al., 2004). In diseases of the central nervous system, mast cells respond to microenvironmental stimuli, modulate inflammation, and contribute to injury by secreting inflammatory mediators. In addition, they also exert a neuroprotective action by secreting proteases associated with the degradation of pro-inflammatory cytokines (Nielissen et al., 2014). Therefore, mast cells, particularly those found in tuberculoid granulomas, might be involved in promoting or attenuating neural inflammation.

The number of eosinophils was negligible in all forms of the disease and types of reaction, which indicates that eosinophils are not involved in the immunopathogenesis of leprosy.

Leprosy is modulated by a number of inflammatory and immunopathological events resulting from the interaction between bacteria, their products, and the host immune response. Our study demonstrates that several types of cells participate in the pathogenesis of leprosy. Furthermore, it contributes to the understanding of this interaction with additional data on: (1) the compartmentalization of macrophage subtypes in TT, LL, R1, and R2 granulomas; (2) the prevalence of Tregs in R1 compared to R1-S patients and a decrease in Tregs in R2 compared to R2-S individuals; (3) the presence of epidermotropic $CD8^+$ cells in the basal layer of the epidermis; and (4) the prevalence of $CD20^+$ B lymphocytes in the tuberculoid type. Therefore, the study of cells with different leprosy lesions is relevant for understanding the mechanisms underlying the progression of the disease and the type of reaction, as well as for investigating new prognostic and predictive factors for both the disease and reactions.

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Authors contributed statement. LRVF conducted the experiments and wrote the manuscript. CTS devised and supervised the study, wrote the manuscript, and conducted the histopathological examination. AFFB, PRS, and APFT contributed to data analysis. APFT provided statistical analysis expertise. CCG conducted clinical diagnosis. MFF supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

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