

# Human mucosal leishmaniasis: Neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines

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Mucosal leishmaniasis (ML) is characterised by severe tissue destruction. Herein, we evaluated the involvement of the IL-17-type response in the inflammatory infiltrate of biopsy specimens from 17 ML patients. IL-17 and IL-17-inducing cytokines (IL-1 $\beta$ , IL-23, IL-6 and TGF- $\beta$ ) were detected by immunohistochemistry in ML patients. IL-17<sup>+</sup> cells exhibited CD4<sup>+</sup>, CD8<sup>+</sup> or CD14<sup>+</sup> phenotypes, and numerous IL-17<sup>+</sup> cells co-expressed the CC chemokine receptor 6 (CCR6). Neutrophils, a hallmark of Th17-mediated inflammation, were regularly detected in necrotic and perinecrotic areas and stained positive for neutrophil elastase, myeloperoxidase and MMP-9. Taken together, these observations demonstrate the existence of Th17 cells in ML lesions associated with neutrophils in areas of tissue injury and suggest that IL-17 is involved in ML pathogenesis.

**Key words:** Human · Mucosal leishmaniasis · Neutrophils · Th17

## Introduction

Mucosal leishmaniasis (ML), a severe chronic disease caused by leishmania protozoa, remains a serious health problem in several parts of the world, including Brazil [1]. ML is at the hyper-responsive end of the spectrum of clinical diseases caused by *Leishmania braziliensis* [1]. Uncontrolled immune responses have been implicated in ML pathogenesis because T lymphocytes from ML patients initiate intense responses (characterised by lymphoproliferation and cytokine production) despite the low number of

parasites in mucosal lesions [2–4]. In addition to Th1 cytokines, TGF- $\beta$  and IL-6 are also produced in ML lesions, but the significance of this finding is poorly understood [5].

Th17 cells participate in inflammatory responses to several human infectious agents [6, 7]. IL-17, the Th17 signature cytokine, induces tissue damage mediated by neutrophil attraction and proteinase release. Neutrophil recruitment mediated by IL-17<sup>+</sup> cells contributes to disease progression in susceptible mouse strains infected with *L. major* [8]. Although the cytokine combination that leads to human Th17 differentiation and maintenance remains controversial, TGF- $\beta$  and IL-6, along with IL-23 and IL-1 $\beta$ , have been implicated in this phenomenon [9, 10].

Recently in human ML, IL-17 expression has been detected [11], but the cell source of this expression has not yet been

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determined. In this study, we expand on the observations reported by Bacellar *et al.* [11] by demonstrating that in addition to Th17 cells, CD8<sup>+</sup> and CD14<sup>+</sup> cells express IL-17. We also detected the presence of neutrophils expressing proteinases in tissue-damaged areas, suggesting a potential function for Th17 cells in ML lesions.

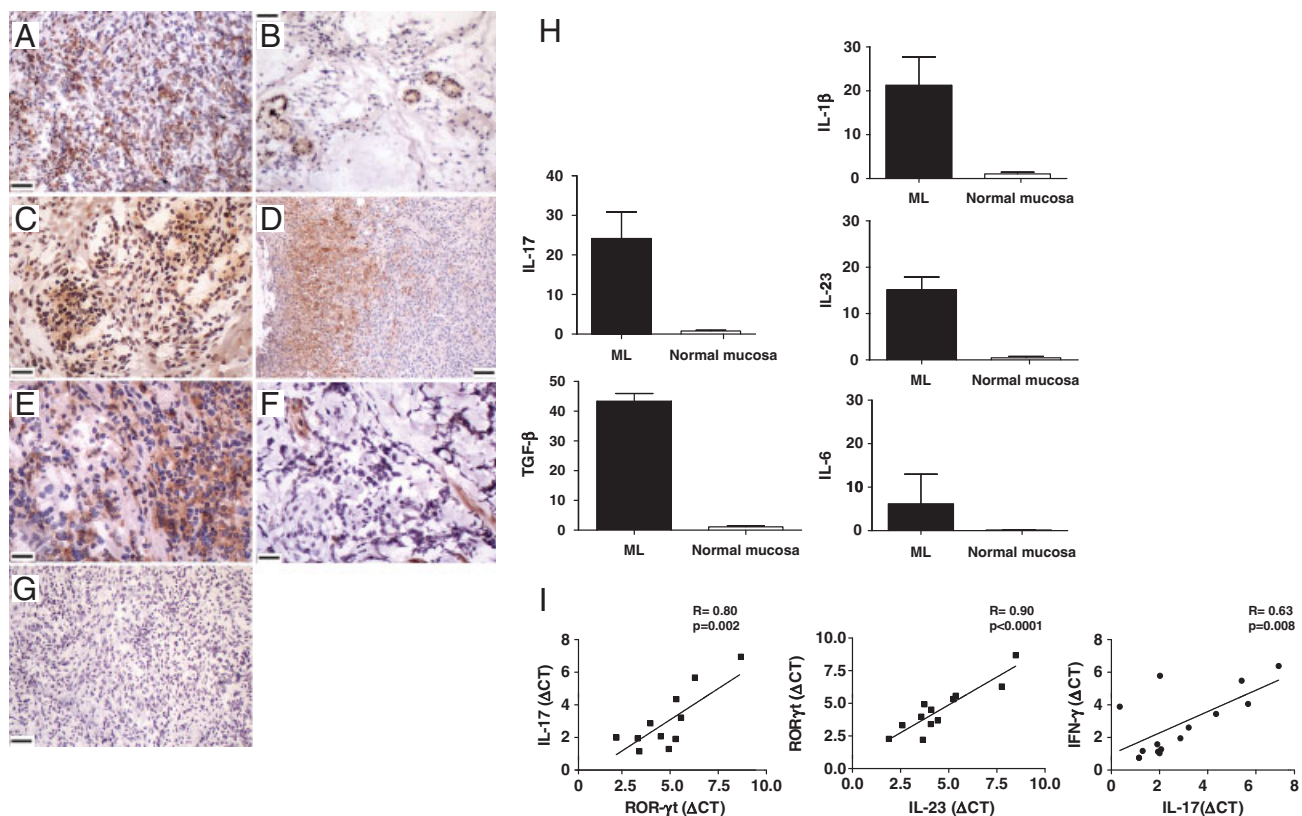
## Results and discussion

### Expression of IL-17, Th17-inducing cytokines and retinoic acid-related orphan receptor $\gamma$ (ROR $\gamma$ t)

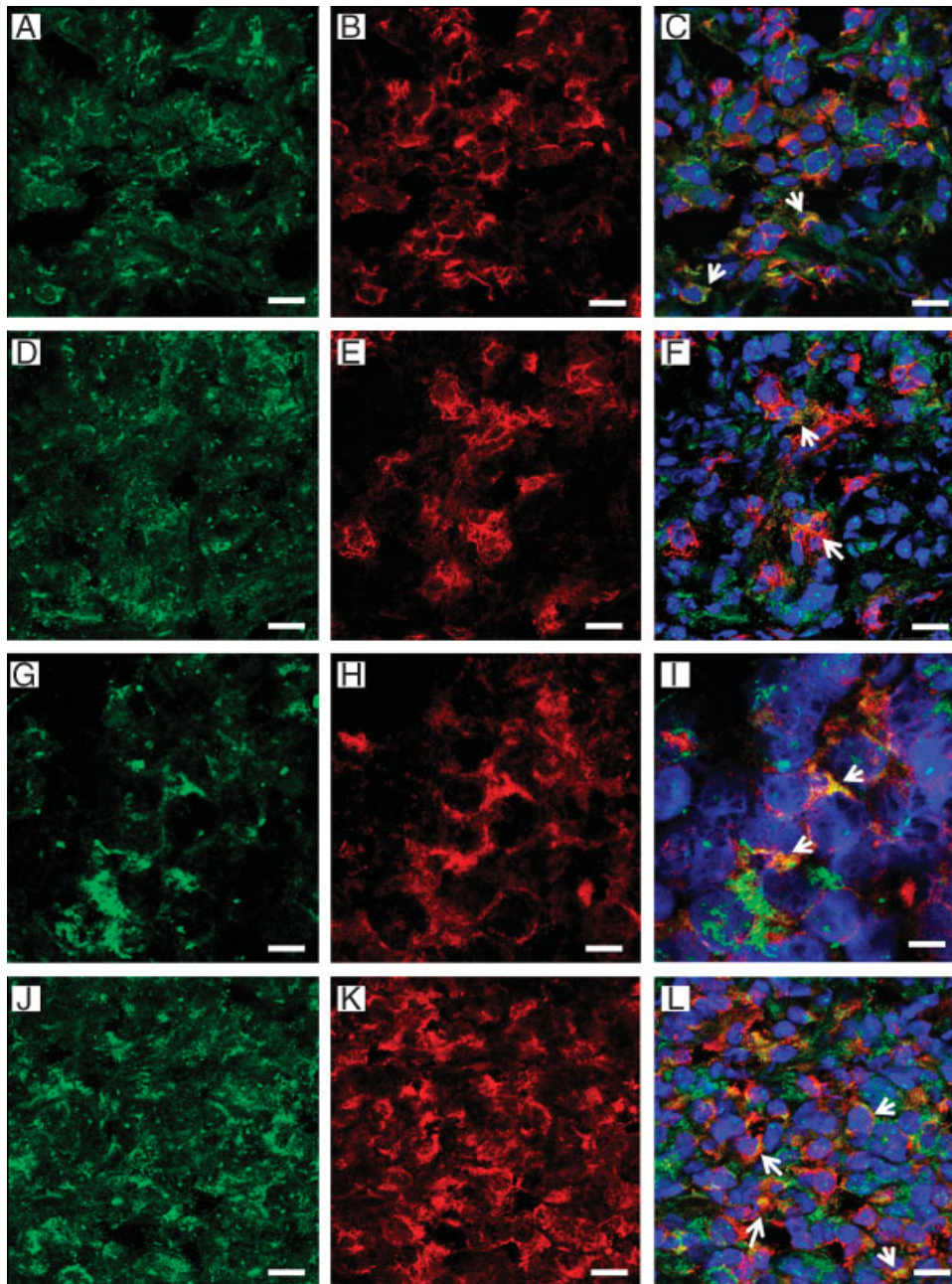
IL-17 expression was consistently higher in ML lesions ( $n = 12$ ) than in normal mucosal samples ( $n = 4$ ), as shown in Fig. 1A and B. Marked expression was detected in mononuclear cells, endothelial cells and perivascular fusiform cells. No reactivity was detected using an isotype control antibody (Fig. 1G). As for cytokines involved in IL-17 production, ML lesions presented an intense expression of both TGF- $\beta$ , which is found in mononuclear cell

aggregates and in endothelial cells disseminated throughout the inflammatory infiltrate (Fig. 1C), and IL-1 $\beta$ , which is detected mainly in mononuclear cells near the ulcer in the inflammatory infiltrate (Fig. 1D). IL-23 was heterogeneously distributed in ML patients, alternating between intense signals in mononuclear cells in some tissue samples (Fig. 1E) and only slight reactivity in other specimens. Weak IL-6 staining was occasionally observed in mononuclear cells located at periglandular areas and in blood vessels dispersed in the inflammatory infiltrate (Fig. 1F). Cytokine quantification analyses revealed higher expression of all cytokines in ML lesions than in normal mucosal tissue samples (Fig. 1H). Therefore, we confirmed that IL-17<sup>+</sup> cells are present in human mucosal lesions caused by *L. braziliensis*. Furthermore, we expanded on results from the previous studies by showing that such cells are present in a cytokine milieu that favours local production of IL-17, as demonstrated by the presence of TGF- $\beta$ , IL-1 $\beta$ , IL-23 and IL-6.

Because IL-17 synthesis requires transcription of ROR $\gamma$ t [8] and IL-23 enhances expression of ROR $\gamma$ t [12], we assessed the mRNA



**Figure 1.** Expression of IL-17, ROR $\gamma$ t and IL-17-inducing cytokines in ML lesions. (A–F) Immunohistochemistry for IL-17, TGF- $\beta$ , IL-1 $\beta$ , IL-23 and IL-6, in ML lesions. (A) Light microscopic analysis of IL-17 immunostaining on a ML lesion with intense staining in the inflammatory infiltrate. (B) Normal mucosal tissue was used as negative control for IL-17 immunostaining. Immunostaining for (C) TGF- $\beta$ , (D) IL-1 $\beta$ , (E) IL-23, (F) IL-6 and with (G) isotype control. All sections were counterstained with hematoxylin. (A, C, E and F) scale bar = 25  $\mu$ m. (B, D and G) scale bar = 50  $\mu$ m. (H) Bars represent quantitative analysis of cytokine stained area in ML ( $n = 5$ ) and normal mucosa ( $n = 3$ ) specimens. Data expressed as percentage of cytokine-positive stained area per total tissue area. Cytokine staining was significantly higher in ML than in normal mucosa  $p < 0.001$  according to ANOVA test. (I) Correlation between transcription of IL-17 and ROR $\gamma$ t (11 samples), IL-23 and ROR $\gamma$ t (12 samples), and IL-17 and IFN- $\gamma$  (14 samples). Correlation coefficient ( $r$ ) calculated by the Spearman's test. The mRNA level represents the average threshold cycle ( $\Delta$ CT), calculated as average cytokine (or ROR $\gamma$ t) threshold cycle – average  $\beta$ -actin threshold cycle.



**Figure 2.** IL-17<sup>+</sup> cell phenotypes. Confocal microscopy images obtained from inflammatory infiltrates of three ML patients. (A, D, G and J) IL-17 staining (green); (B) CD4 staining (red); (E) CD8 staining (red); (H) CD14 staining (red); (K) CCR6 staining (red). (C, F, I and L) Overlays show production of IL-17 in CD4<sup>+</sup> cells (arrowheads in (C), yellow), in CD8<sup>+</sup> cells (arrowheads in (F), yellow), in CD14<sup>+</sup> cells (arrowheads in (I), yellow) and in CCR6<sup>+</sup> (arrowheads in (L), yellow). Data are representative of results from three ML patients. (A–F) and (J–L): scale bar = 10  $\mu$ m, (G–I) scale bar = 5  $\mu$ m.

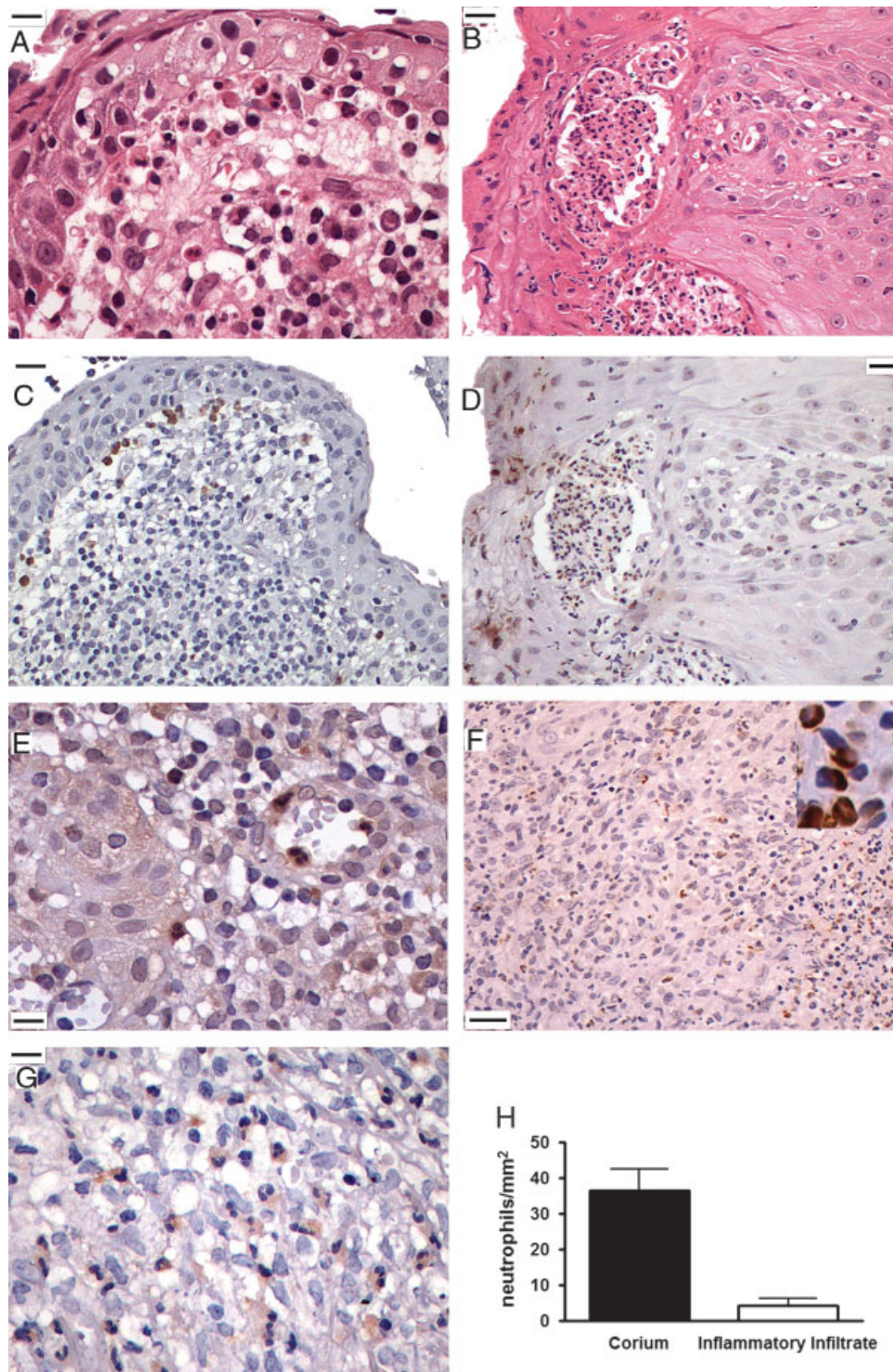
expression of IL-17, ROR $\gamma$ t and IL-23 in ML lesions using real-time PCR. A positive correlation between the expression of mRNA for IL-17 and ROR $\gamma$ t, as well as between ROR $\gamma$ t and IL-23 transcripts existed in ML patients (Fig. 1I). We also detected a positive correlation between the expression of IL-17 and IFN- $\gamma$  mRNA in ML lesions (Fig. 1I). Flow cytometric analysis revealed that about 3% of mucosal lesion cells express either IFN- $\gamma$  or IL-17, but less than 0.5% co-express IFN- $\gamma$  and IL-17 (data not shown). In addition to the previously described roles of Th1 clones and the critical effector

cytokine IFN- $\gamma$  in ML pathogenesis [5], these cells are involved in Th17 recruitment to tissue lesions. For example, the recruitment of Th17 cells is stimulated by a Th1 clone in psoriatic lesions [13]. In this circumstance, Th1 and Th17 cells act together to induce immune-mediated tissue damage. Furthermore, IL-17, in association with Th1 cytokines, plays a protective role in human visceral leishmaniasis, a lethal disease characterised by intense parasite proliferation [14]. Th17 cells also participate in the host defence against extracellular bacterial and fungal pathogens, such as



*Listeria monocytogenes*, *Salmonella enterica*, *Mycobacterium tuberculosis* and *Candida albicans* [15]. Whether Th17 cells play a

protective or a pathogenic role in ML infection requires further investigation.



**Figure 3.** Neutrophil infiltration and proteinase expression in ML lesions. (A) Neutrophils infiltrating the subepithelial area. (B) Neutrophils in intraepithelial pustules. (C and E) NE immunostaining in neutrophils from epithelium and lamina propria. (D) MPO immunostaining in intraepithelial pustules. (F) MPO immunostaining in necrotic and perinecrotic areas. Inset: MPO staining in neutrophils. (G) MMP9 staining in neutrophils. (A, E and G) scale bar = 10  $\mu$ m. (B, C and D) scale bar = 20  $\mu$ m. (F) scale bar = 25  $\mu$ m. (H) The proportion of neutrophils was significantly higher in corium areas than in the inflammatory infiltrate ( $n = 4$ ,  $p < 0.05$ ). All sections were counterstained with hematoxylin. Isotype control antibodies were used as negative control for all immunostaining.

## IL-17 is co-expressed by CD4<sup>+</sup>, CD8<sup>+</sup>, CD14<sup>+</sup> and CC chemokine receptor 6<sup>+</sup> cells

We further investigated the cell sources of IL-17 (Fig. 2). The percentages of CD4<sup>+</sup>, CD8<sup>+</sup> and CD14<sup>+</sup> cells in ML lesions were, respectively,  $56.3 \pm 10$ ,  $18.5 \pm 2.1$  and  $47.2 \pm 10.7$ , when evaluated by confocal microscopy. CD4<sup>+</sup> (Fig. 2C), CD8<sup>+</sup> (Fig. 2F) and CD14<sup>+</sup> (Fig. 2I) cells all co-stained with IL-17. The frequencies of double-positive cells expressing CD4/IL-17, CD8/IL-17 and CD14/IL-17 within single CD4<sup>+</sup>, CD8<sup>+</sup> and CD14<sup>+</sup> cells were  $34.6 \pm 2$ ,  $21 \pm 1.4$  and  $62.6 \pm 10.2$ , respectively. No significant IL-17 staining was detected in normal mucosa or normal skin specimens (data not shown). CD14 is expressed mainly by macrophages but can also be produced by neutrophils or dendritic cells. However, few CD14<sup>+</sup> cells were detected by confocal microscopy or flow cytometric analysis (data not shown), suggesting that they make only a small contribution to IL-17 expression in ML lesions.

CD8<sup>+</sup> T cells have been recognised as important components of the cellular immune response to leishmania via IFN- $\gamma$  production and parasite-driven cytotoxicity [4–16]. The local detection of CD8<sup>+</sup>IL-17<sup>+</sup> cells is of particular interest since a noncytotoxic 17 (Tc17) CD27<sup>+/-</sup> CD28<sup>+</sup> CD45RA<sup>-</sup> subset has recently been described in other inflammatory diseases [17]. Such cells express CC chemokine receptor 6 (CCR6) and produce IFN- $\gamma$  [17]. In addition, tissue-infiltrating IL-17<sup>+</sup> cells co-express CCR6 (Fig. 2L), a chemokine receptor known to be present in neutrophils and Th17 cells and related to the migration of these cells to inflamed tissues. CCR6<sup>+</sup> cells producing IL-17 within the lesion site corroborate the hypothesis that Th17 migration is mediated by this chemokine receptor [18].

## Neutrophils infiltrate ML lesions and express proteinases

Because Th17-driven inflammation is classically characterised by neutrophil infiltration [19], we investigated the presence of these cells in ML lesions. Neutrophils were observed in all ML lesions with varying frequency between patients. Neutrophils were concentrated mainly in the epithelium and lamina propria, at the edges of ulcerous or necrotic areas ( $36 \pm 26$  cells/mm<sup>2</sup>, Fig. 3A and H). Their density was much lower in the deeper portions of the chronic inflammatory infiltrate, where only a few isolated neutrophils were observed ( $4 \pm 8$  cells/mm<sup>2</sup>, Fig. 3H). Superficial erosions are frequently detected in this area during clinical examination of ML patients, which may be related to neutrophil infiltration and expression of proteinases. In 30% of ML specimens, large numbers of neutrophils were observed inside dilated capillaries only. In two patients, neutrophil aggregates were observed in intraepithelial pustules (Fig. 3B and D). Moreover, neutrophils exhibited intense immunostaining for neutrophil elastase (NE; Fig. 3C and E), myeloperoxidase (MPO; Fig. 3D and F) and MMP9 (Fig. 3G).

Assuming that Th17 cells mediate neutrophil infiltration in ML lesions, IL-17 may favour inflammatory immune responses in ML

patients by recruiting neutrophils. In experimental cutaneous leishmaniasis, lesion progression is related to IL-17-mediated neutrophil recruitment, whereas improved disease outcome is associated with decreased neutrophil immigration in IL-17-deficient mice [8]. However, neutrophils contribute to parasite clearance in the early steps of experimental leishmania infection [19] and activate macrophages to kill *L. major* by a mechanism that requires NE [20]. Thus, the presence of neutrophils in this inflammatory context can be indicative of either protection or injury.

## Concluding remarks

Taken together, the data presented here demonstrate that Th17 conditions, as well as CD8<sup>+</sup> and CD14<sup>+</sup> cells expressing IL-17, participate in the inflammatory response to ML. Neutrophil chemotaxis with proteinase release in ML lesions in damaged areas can be mediated by Th17 cells. Investigating the role of Th17 cells in ML may lead to new strategies of enhancing protective immunity and minimising immune response-mediated tissue damage during this disease.

## Materials and methods

Tissue samples were obtained from 17 ML patients (1.4 male/female ratio, aged  $59 \pm 17$  years) at our field clinic in the Bahia State, Brazil. ML diagnosis was confirmed by the presence of a granulomatous mucosal lesion associated and at least one of the following tests: positive anti-leishmania delayed type hypersensitivity, detectable anti-leishmania antibody titres or the presence of leishmania in the biopsy tissue as detected by either immunohistochemistry or PCR. All patients had experienced symptoms for a prolonged time period (mean time of disease  $10 \pm 14$  years) and presented with mucosal lesions involving the nasal cavity (100%), pharynx (35%) and/or larynx (11%). All tissue specimens were obtained before treatment; afterwards, patients received *N*-methylglucamine antimoniate (20 mg/Sb/kg/d) for 30 days. Nasal mucosal biopsy was performed under local anaesthesia with Lidocaine<sup>®</sup> spray (10%). Normal mucosal samples were obtained from turbinectomy nasal surgery. Tissue fragments were cryopreserved or conserved in 10% formalin. This study was approved by the Gonçalo Moniz Research Center (CPqGM/FIOCRUZ-Bahia) Institutional Review Board, and informed consent was obtained from all patients before enrolment.

## Immunohistochemistry staining

Frozen sections (5  $\mu$ m thick) were obtained and immunohistochemistry was performed as described previously [2]. The following primary antibodies were used: rabbit anti-IL-17 (4  $\mu$ g/mL) or anti-TGF- $\beta$  (2  $\mu$ g/mL) (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-IL-23 (0.01  $\mu$ g/mL), mouse



anti-IL-6 (25 µg/mL), mouse anti-IL-1β (10 µg/mL) or goat anti-MMP-9 (4 µg/mL) (all R&D Systems, Abingdon, UK), goat anti-MPO (4 µg/mL; US Biological, Swampscott, MA, USA) and goat anti-NE (12 µg/mL; Santa Cruz Biotechnology). Biotin-labelled anti-rabbit, anti-mouse or anti-goat IgG (Vector Laboratories, Peterborough, England) was used as a secondary antibody. Isotype control antibodies (R&D Systems) were used as negative controls. Positive-control sections consisted of frozen mucosal tonsillar tissue and frozen nasal polyps. Digital images of tissue sections were captured using a Nikon E600 light microscope and a Q-Color 1 Olympus digital camera. Quantification of stained areas was performed using Image Pro-Plus software (Media Cybernetics).

### Confocal microscopy

Double immunofluorescence staining was performed for IL-17 and CD4, CD8, CD14 or CCR6 markers. The following primary antibodies were used: mouse anti-CD4 (BD Biosciences, San Jose, CA, USA), mouse anti-CD8 (BD Biosciences), mouse anti-CCR6 (R&D Systems) and rabbit anti-IL-17 (8 µg/mL, Santa Cruz Biotechnology). Secondary antibodies were biotin anti-mouse IgG (Vector Laboratories) or anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR, USA). Streptavidin Cy3 (Sigma, Buchs, Switzerland) was used after biotin antibodies. Multiple images representing positive staining and negative controls were acquired using a confocal microscope (Leica TCS SP2 SE and SP5 AOB5). Image Pro Plus was used for image processing.

### Real-time PCR

The extraction of total RNA from mucosal tissues was performed following the protocol recommended by the manufacturer (Life Technologies, Rockville, MD, USA). cDNA was synthesised using 1 µg of RNA through a reverse transcription reaction (M-MLV reverse transcriptase, Promega, Madison, WI, USA). Real-time PCR quantitative mRNA analyses were performed on the ABI Prism 7000 Sequence Detection System. Nucleotide sequences of human primers are present in the GenBank database. The SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.1–0.2 µg/µL specific primers, and 2.5 ng of cDNA were used in each reaction. Calculations to determine the relative level of gene expression were made according to the manufacturer's instructions, with reference to the β-actin in each sample, using the cycle threshold method. Negative controls without RNA and without reverse transcriptase were included.

### Statistical analysis

The ANOVA test was used to compare stained areas in the immunohistochemistry assay. Differences in neutrophil numbers were analysed using the Mann–Whitney *U*-test. Correlation

analyses were performed by Spearman's test. A *p*-value less than 0.05 was considered significant. Statistical analysis was performed using Prism 4 software (GraphPad Software, San Diego, CA, USA).

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**Conflict of interest:** The authors declare no financial or commercial conflict of interest.

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**Abbreviations:** CCR6: CC chemokine receptor 6 · ML: mucosal leishmaniasis · MPO: myeloperoxidase · NE: neutrophilic elastase · ROR $\gamma$ t: retinoic acid-related orphan receptor gamma t

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