Journal Pre-proof

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Title Page

Selective suppression of cellular immunity and increased cytotoxicity in skin lesions of disseminated leishmaniasis uncovered by transcriptome-wide analysis

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Short title: RNA-seq of disseminated leishmaniasis lesions

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Abbreviations

CL (cutaneous leishmaniasis), DCL (diffuse cutaneous leishmaniasis), DL (disseminated leishmaniasis), LCL (localized CL)

Letter

To the Editor:

Disseminated leishmaniasis (DL) is an emerging form of cutaneous leishmaniasis (CL) where multiple acneiform or papular lesions occur at ≥ 2 noncontiguous anatomical regions affecting various body segments (Costa et al. 1986). With increasing incidence in endemic areas of *L. braziliensis* transmission, DL is a difficult-to-treat disease due to the elevated number of lesions, the frequent mucosal involvement and the low cure rate (Machado et al. 2019). Lack of lymph node enlargement and the rapid spread of lesions to many parts of the skin and mucosa suggest that parasites disseminate via the bloodstream (Costa et al. 1986), and both host and parasite factors have been implicated in this manifestation. DL patients exhibit an in situ response characterized by IFN- γ , TNF, IL-10, TGF β , CCL2, CCL3, and CXCL10, with levels similar to those observed in localized CL (LCL) lesions (Machado et al. 2011). However, the host response in DL has so far only been studied by using limited cytokine panels, and more comprehensive studies are needed.

To investigate the transcriptome-wide in situ response in DL, we employed RNA sequencing of lesion biopsies (n=7) and compared to unrelated healthy skin controls (n=4); institutional approval for the experiments and written informed patient consent were obtained (Table S1; Supplementary Material and Methods). 2,149 differentially expressed genes (DEGs) were identified in this comparison (Table S2), the majority of which were upregulated (1,765 genes) while only 384 were found to be downregulated (Figure S1). Enrichment analysis of these DEGs was performed, and a total of 49 processes were found to be upregulated, whereas eight were downregulated (Figure S2a). Collectively, these results indicate that gene expression in DL is geared towards activation of tissue remodeling pathways, with concomitant dampening of the cellular immune response, a hallmark of LCL (Figure S2a, S2b). We then further explored which markers of the immune response

characterized this observed deregulation, with respect to the relevance of CD4⁺ T-cells and IFN- γ in controlling *Leishmania* infection (reviewed in Novais and Scott, 2015). In general, transcripts coding for inflammatory cytokines, including IL1B, and IL6, were more expressed in DL lesions than controls, whereas *LTA* and *TNF* were downregulated (Figure 1a). Of note, the expression of *IFNG* did not reach statistical significance compared to controls, although it was found more expressed in some of the lesions ($\log_2FC=2.66$; $FDR=0.12$) (Figure 1a). Strikingly, chemokines and markers of cytotoxicity were strongly upregulated, which is consistent with the observed enrichment of biological processes related to cell chemotaxis and inflammatory response (Figure S2a). Upon examination of the number of transcripts expressed by each patient, the significantly higher expression of *IL10* (associated with parasite persistence), confirmed by immunohistochemical staining, in parallel with markedly lower expression of *IL13*, *LTA*, *TNF* and *TGFB1* were notable (Figure 1b, 1e). Variability in the expression of *IFNG* and *IL10* has been reported in previous transcriptomic studies using lesions, with *IFNG* ranging between 3.12–8.29 and *IL10* within 0.5–4.54 ($\log_2FC$ vs. controls) in LCL datasets (Novais et al. 2015; Christensen et al. 2016; Amorim et al. 2019; Rodrigues et al. 2019). Thus, the expression profiles of *IFNG* and *IL10* identified in DL lesions lay within the lower range of expression compared to LCL forms. Of note, diffuse CL (DCL) also presents with lowered *IFNG* production *in situ* that, together with other unique features of this manifestation which also involves parasite spread, could be associated with a shift from Th1 responses impairing a proper host immune defense (Christensen et al. 2019). As expected, the number of transcripts coding for chemokines was significantly higher in DL lesions compared to controls (Figure 1c). Lastly, we observed a significant increase in *GZMA*, a T- and NK-cell-specific serine protease associated with cytotoxic response. Other mediators of cytotoxicity were also significantly upregulated in DL lesions: cathepsin-B (*CTSB*), granzyme-H (*GZMH*), cathepsin-C (*CTSC*) and FAS ligand (*FASLG*) (Table S2).

The notion of an impaired type 1 immune response in DL patients was previously proposed based on the low expression of IFN- γ and TNF- α in PBMC (Leopoldo et al. 2006; Machado et al. 2011), but no differences in their in situ production were observed between DL and LCL samples by Machado et al. (2011). Importantly, the Th1/Th2 paradigm observed in experimental infection models, wherein the first associates to cure and the latter to disease progression (Sacks and Noben-Trauth 2002), does not fully capture the multiple facets of the disease in humans, which is characterized by stark heterogeneity in clinical presentations and, not uncommonly, by mixed Th1/Th2 immune responses. Collectively, these results strengthen and expand the notion that selective immunosuppression, coupled with activation of cytotoxic molecules, is present in DL lesions.

Next, we compared the transcriptome-wide signature between DL and previously published lesion transcriptomes of patients with LCL (Christensen et al. 2016; Novais et al. 2015) and DCL (Christensen et al. 2019). In DCL, pathogen spread is accompanied by an abundance of parasites (unlike DL) and patients are refractory to treatment (Silveira et al. 2004). GSEA analysis using MSigDB as a template disclosed unique/shared pathways among these forms, six of which common to all conditions, and 25 being DL-specific (Figure 2). Identified in the core response are pathways related to inflammation, a prominent feature of tegumentary leishmaniasis, while processes related to signaling, development, and proliferation were exclusively recognized in DL, including MYC targets, the p53 pathway, signaling by KRAS, hedgehog and TGF beta, as well as angiogenesis (Figure 2), in support of previous findings suggesting a role for vessel proliferation to parasite survival and growth in DL (Mendes et al. 2013). These multiple biological processes potentially contribute to driving more research dissecting the basis of parasite dissemination in this clinical manifestation.

To explore the population of cells that could be involved in the immune landscape described above, digital cytometry (Finotello et al. 2019) was performed using RNA

sequencing data as input. DL lesions were characterized by the presence of neutrophils ($11.1 \pm 4.1\%$; mean $\pm$ s.d. of quanTIseq-predicted cell fractions), NK cells ($6.1 \pm 1.7\%$), M1 ($13.6 \pm 8.4\%$) and M2 (3.1 ± 2.0) macrophages (**Figure S3a**). Other cell populations were also detected, such as $CD4^+$ and $CD8^+$ T-cells and B-cells, albeit with variability that likely reflects interspecimen heterogeneity. For instance, the estimated abundance of B-cells in one patient was 29% (range: 3–29%; mean $\pm$ s.d. of $7 \pm 10\%$). Coincidentally, the two patients that presented the highest proportions of B-cells (29% and 8%) also had mucosal involvement. Previously reported evidence in human CL forms caused by *L. braziliensis* showed that overexpression of B-cell transcripts occurs, to varying degrees, in lesions (Christensen et al., 2019; Amorim et al., 2019), and B-cells were also identified in DL lesions (Mendes et al. 2013). Taken together, our findings suggest that DL lesions can be characterized by an inflammatory signature in which chemokines, neutrophils and macrophages are strongly present, in addition to cytotoxic markers and NK cells. To assess the differences in the immune infiltrate composition across various CL manifestations, we also compared the prediction of the immune infiltrate of DL lesions with those of DCL and LCL. These results revealed unique patterns of each disease (**Figure S3a**), as well as marked variability within CL presentations (**Figure S3b**). Notably, DL and DCL presented with significantly lower estimates of M1 macrophages in situ, and these classically-activated macrophages are usually associated with *Leishmania* clearance (Christensen et al. 2019). The presence of $CD4^+$ and $CD8^+$ cells was confirmed by immunohistochemistry in independent samples of DL and LCL lesions, with DL presenting a weaker signal of both T-cells subsets (**Figure S3c**). Decreased $CD8^+$ cell fractions were also detected in DL samples compared to LCL (**Figure S3a**), but this was not statistically supported. However, lower fractions of $CD8^+$ in DCL samples, which we verified using digital cytometry (**Figure S3a**), have also been reported in the literature (Hernández-Ruiz et al. 2010). In humans, recruitment of granzyme-producing $CD8^+$

T-cells to lesions is associated with CL severity, while also contributing to tissue damage in cutaneous lesions caused by *L. braziliensis* (Brodskyn et al. 1996; Santos et al. 2013). In mice, cytotoxic T-cells promote metastatic lesions (Novais et al. 2013). If further studies confirm a lower number of CD8⁺ T-cells in DL lesions, this could point to NK cells as a source of cytotoxicity (Berke 1995), a finding recently reported in the PBMC of aggravated LCL patients (Campos et al. 2020).

Our study presents possibilities and limitations of the transcriptome-wide analysis of human CL lesions. Particularly, our unpaired study design is unable to fully account for individual-level variations in gene expression, which could be overcome by a paired design with control samples deriving from healthy, and preferably comparable, skin segments of the same subject, yielding an improved statistical power. However, bioethical concerns in performing a punch biopsy in the healthy skin of a patient prompted us to employ alternative control skin obtained from surgical discards of individuals undergoing elective procedures. Although our limited sample size narrows the ability to draw definitive conclusions, particularly concerning the prediction of cellular populations in DL lesions, we were able to validate *in silico* predictions of T-cells subsets by immunohistochemistry.

In sum, this analysis revealed that DL is a manifestation that differs from other CL forms clinically and also at the gene expression level. Our results not only highlighted similarities across the spectrum of human CL, but revealed specific markers to each clinical form, which may ultimately be reflected by differences in host gene expression levels during active disease. Functional studies are warranted to follow-up on some of the unique molecular pathways identified in this work, contributing to an enhanced comprehension of the mechanisms by which parasite dissemination occurs in DL, and how to prevent it.

Data availability

Datasets related to this article can be found in NCBI's Sequence Read Archive (Leinonen et al. 2011), and are accessible through BioProject accession PRJNA576449.

Conflict of Interest

The authors state no conflict of interest.

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Author Contributions

Conceptualization: AB, MB-N

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Performed molecular and cellular experiments: CRBC, CFP, JRC, JVW, RS

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Writing – Original Draft Preparation: CIO, PIPR

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Figure Legends

Figure 1

Bird's-eye view of the modulation of selected immune-related genes in disseminated leishmaniasis lesions.

a) Circular plot linking immune response processes with corresponding gene expression. Link colors indicate more (red) or less (blue) expression in DL compared to controls, or no gene modulation (gray). Color of nodes near gene labels reflects log₂-transformed fold-change values. Differentially expressed genes appear bolded. **b-d)** Normalized expression values of genes that appear in panel **a**. Data are expressed as mean+SEM for n=7 (DL group) and n=4 individuals (control group), with individual data points overlaid. DL and control groups were compared using the Mann-Whitney U-test. *P-value<0.05, **P-value<0.01. The representative transcripts analyzed here were obtained from previous studies (Christensen et al. 2019 and references therein). **e)** Immunostaining of IL-10 in tissue sample from DL lesion and normal skin obtained from plastic surgery. Magnification 40x, scale bar = 50 μ m.

Figure 2

Conserved and shared transcriptional patterns across the spectrum of tegumentary leishmaniasis.

GSEA was performed for available expression data of lesions of three clinical forms of ATL: DL (this work), DCL (Christensen et al. 2019) and LCL (Novais et al. 2015; Christensen et al. 2016). Circles represent activated hallmark gene-sets (NES>0) in the comparison of infected vs. healthy skin for each dataset, with radii proportional to NES. Circle colors

represent FDR-corrected P -values for each term, with gray-colored circles indicating terms that, although enriched in a given dataset, had $\text{FDR} > 0.05$. Hallmark processes were considered shared between LCL and other clinical forms when significantly enriched in at least one LCL dataset.

Supplementary files

Supplementary Material and Methods

Extended description of the Material and Methods.

Figure S1

Volcano plot showing differentially expressed genes in DL versus healthy skin biopsies. Each dot represents a gene, and colors denote genes that: were not found differentially expressed (black dots); were significant based on q -value but with $\log_2\text{FC}$ in the $[-3, 3]$ interval (gray dots); were significantly upregulated (red dots); or were significantly downregulated genes (blue dots). The number of genes in a given class is shown in red at the corresponding quadrant, except those that did not reach statistical significance nor a minimum fold-change deviation. q -value, FDR-adjusted P -value.

Figure S2

Enriched and depleted biological processes in disseminated leishmaniasis.

a) Forty-nine processes were significantly enriched in DL compared to healthy skin samples. Circle size is proportional to the number of genes in each respective process, and color varies according to the degree of enrichment/depletion (hypergeometric test). **b, c)** Detailed hierarchical view of the enriched (panel **b**) and depleted (panel **c**) processes, with related

terms grouped in same-colored rectangles. Rectangle size reflects process enrichment. Summarization performed using REVIGO (see Supplementary Material and Methods).

Figure S3

Digital cytometry of DL, DCL and LCL lesions evidences unique patterns within each clinical forms, as well as marked interindividual differences

quantIseq was used to perform digital cytometry using RNA-seq data. The fractions of predicted immune infiltrate were compared across lesions of DL (this work), DCL (Christensen et al. 2019) and LCL (Christensen et al. 2016; Amorim et al. 2019). **a)** Summarization of the results of immune cell populations shown as boxplots. Overlaid black points represent cell population estimates in individual samples. The Mann-Whitney U-test was applied to compare groups. * P -value<0.05, ** P -value<0.01, *** P -value<0.001, **** P -value<0.0001. B, B cells; CD4, total CD4⁺ T cells (including also CD4⁺ regulatory T cells); CD8, CD8⁺ T cells; DC, dendritic cells; M1, M1 macrophages; M2, M2 macrophages; NK, natural killer cells. **b)** Individual composition of the predicted immune infiltrate in each analyzed skin lesion. **c)** Immunohistochemical staining of CD4⁺ and CD8⁺ cells in biopsy samples from skin lesions of patients with DL or LCL, counterstained with hematoxylin. Magnification 40x, scale bar = 50 μ m.

Table S1

Demographic and clinical features of individuals enrolled in the study

Table S2

Complete differential expression dataset (DL vs. controls) for the processed RNA-seq experiment, showing all 15,717 host genes that appeared expressed in the studied samples.

Annotation, $\log_2$ -fold-change, and normalized expression (cpm) values are shown with corresponding FDR-corrected P -values. Significantly up- and downregulated genes (criteria described in Supplementary Material and Methods) for the comparison DL vs. control groups appear in a separate sheet.

a **c** **b**

Supplementary Material and Methods

Selective suppression of cellular immunity and increased cytotoxicity in skin lesions of disseminated leishmaniasis uncovered by transcriptome-wide analysis

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Ethics statement

This study was conducted in Mutuípe, Bahia, Brazil, an area where cutaneous leishmaniasis, caused by *L. braziliensis*, is endemic. The present research protocol received approval from the Institutional Review Board of Gonçalo Moniz Institute (IGM-FIOCRUZ) (protocol no. 1.673.870/2015-CEP/CPqGM/FIOCRUZ) and the Brazilian National Ethics Committee (Comissão Nacional de Ética em Pesquisa, CONEP). Informed written consent was obtained from each participant. All methods were performed in accordance with the guidelines and regulations determined by the CONEP.

Study design and patient enrollment

Individuals diagnosed with DL were enrolled in the study ($n = 7$) and subjected to RNA sequencing from lesions. Inclusion criteria consisted of: The presence of 10 or more cutaneous acneiform lesions located at two or more body segments, and a positive Montenegro Skin Test and/or histopathological examination. Individuals 18 years or older were invited to participate. Exclusion criteria consisted of immunocompromised individuals, pregnancy, or the presence of atypical disease forms. Controls for the transcriptome-wide study consisted of healthy skin samples ($n = 4$) obtained from donors subjected to elective procedures (three male samples from penile foreskin excisions and one female sample from mammoplasty). Skin sample collection resulted in no alterations to surgical procedures, and all samples were routinely removed according to the originally planned procedure.

Montenegro skin test

Skin test using *Leishmania* antigen was performed after 48 to 72h post intradermal inoculation using a ruler. Inoculation of 1 mL of soluble *Leishmania* antigen (obtained from Fundep/UFMG/BH/MG) was performed using a tuberculin syringe in the anterior face of the

right forearm. Results were interpreted based on the area of skin induration, with values smaller than 25 mm² considered negative, and those greater than 25 mm² as positive reaction (Mayrink et al. 1993).

Lesion selection and biopsy procedures

Lesions were selected for biopsy procedure according to their clinical aspect. Biopsy was performed in the border of an active cutaneous lesion (early, elevated, ulcerated or pre-ulcerated). After local anesthesia with 2% lidocaine, biopsies were collected at the border of DL lesions using a 4 mm punch prior to the initiation of therapy and divided into two fragments: one was maintained in formaldehyde and processed for histological analysis, while the other was kept in *RNAlater* (Ambion, Austin, TX, USA) at -70 °C until the time of RNA isolation. Skin biopsies from healthy controls were processed similarly.

RNA isolation and cDNA library preparation

The cryopreserved biopsies were processed into fine powder using the traditional mortar and pestle system under liquid nitrogen. Total RNA was extracted from these samples using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and DNA clean up was performed on-column by DNase treatment (Qiagen) in accordance with the manufacturer's recommendations. RNA was quantified on a NanoDrop spectrophotometer (Thermo Scientific, Nanodrop 1000) and RNA integrity was assessed using an Agilent Bioanalyzer (Agilent Technologies), yielding an RNA integrity number (RIN) for each sample. One out of eight RNA samples of DL patients were excluded because of low quality (RIN <2), and the seven remaining samples had a median RIN value ($\pm$ standard deviation) of 7.0 ± 0.25 . RNA was frozen in -80 °C until the time of library construction. rRNA was depleted from total RNA using the Eukaryote RiboMinus kit (Invitrogen). RNA-seq libraries were prepared

using the Illumina TruSeq Stranded kit (Illumina, San Diego, CA, USA) as per manufacturer's instructions. Libraries were then sequenced on an Illumina NextSeq500 instrument (Illumina, San Diego, CA, USA) using the Illumina NextSeq500 High Output kit, producing 150 bp paired-end reads.

Immunohistochemistry

Immunohistochemistry staining was performed in fresh-frozen specimens obtained by biopsy from four patients with CL and two of DL. We also selected samples from healthy donors submitted to plastic surgery for aesthetic reasons (Local Human Ethics Committee - protocol number 3.334.252). These samples were independent from those that underwent RNA sequencing. All samples were embedded in tissue-tek O.C.T. compound (Sakura Finetek USA) and snap-frozen in liquid nitrogen.

Cryostat-frozen sections, 5 μ m thick, were applied to microscope slides and fixed with cold acetone. Staining was performed with rat polyclonal anti-CD4 (cat. n. 13573), anti-CD8 (cat. n. 59130), or goat polyclonal anti-IL-10 (cat. n. 1783) antibodies (Santa Cruz Biotechnology, USA), followed by secondary biotinylated anti-rat or anti-goat antibodies (Vector Laboratories, USA). An avidin-biotin peroxidase system (Vector Laboratories, USA) was used to reveal the reactions and the slides were counterstained with Mayer's hematoxylin.

Data analysis

Sequences were quality-controlled (QC) using Trimmomatic, which also allowed for adapter trimming (Bolger et al. 2014). FastQC was used to calculate various read quality metrics [<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>]. After performing QC, transcript expression was quantified by the Salmon tool (Patro et al. 2017). For this step, the

human reference transcriptome from the GENCODE consortium (release 28) was used to construct a proper index against which quasi-mapping was performed (Harrow et al. 2012). Once transcript expression estimates were calculated, gene-level summarization was conducted using the *tximport* package for R (Soneson et al. 2015) and further gene-level analysis was performed using count-based statistical inference tools. Trimmed mean of M-values (TMM) normalization was performed to obtain normalization factors across samples and to account for underlying RNA composition (Robinson and Oshlack 2010). Prior to testing for differential expression, low-expression genes (*i.e.* those having less than 3 counts per million [cpm] in at least four samples) were filtered. This threshold was chosen to attain a raw read count of approximately 10, based on the depth of the least sequenced library (Chen et al. 2016). Then, edgeR was used to perform differential expression analysis between DL and control samples (Robinson et al. 2010). *P*-values were adjusted for multiple hypothesis testing using a False Discovery Rate (FDR) threshold: Genes were considered differentially expressed when FDR-corrected *P*-value $\leq 10^{-3}$, and $\log_2$ -transformed absolute fold-change ≥ 3 . Enrichment analysis was performed with DAVID 6.8 (Huang et al. 2009b; Huang et al. 2009a) using the DEGs as input and the Gene Ontology (GO) database as an annotation source. GO terms with FDR-corrected *P*-values ≤ 0.05 were considered significant. The REVIGO platform (Supek et al. 2011) was used to remove redundancy in the list of GO terms, employing the SimRel semantic similarity measure with a similarity parameter of 0.7. A comparison of DL with other clinical forms of tegumentary disease was also performed using previously published datasets, namely an RNA-seq analysis of DCL (Christensen et al. 2019) and two LCL datasets generated using a microarray (Novais et al. 2015) and RNA-seq (Christensen et al. 2016). For this, all three previously published datasets, derived from transcriptomic analysis of leishmaniasis skin biopsies compared to healthy donors, as well as the present dataset, were jointly re-analyzed in the Gene Set Enrichment Analysis (GSEA)

framework (Subramanian et al. 2005). Reference gene sets from MSigDB hallmark processes were used during this analysis, derived through the aggregation of multiple MSigDB gene sets to represent well-defined biological states or processes (Liberzon et al. 2015). GSEA was run in pre-ranked mode, and an ordered gene list was produced per dataset by scoring each gene according to the following formula:

where FDR is the False Discovery Rate-corrected P-value from the differential expression analysis for a given gene, and FC is the corresponding fold-change value derived from comparisons between infected and control groups. Duplicated probes in the Novais et al. dataset were collapsed through the maintenance of each gene's maximum score. One thousand permutations of the gene sets were performed to calculate FDR, and a cutoff of 0.05 was used to define significance.

To quantify parasite transcripts in DL lesions, HISAT v. 2.1.0 (Kim et al. 2015) was initially used to map the paired-end reads of each sample against the human genome. Alignment parameters were set at default except for the flag `--un-conc`, which was enforced to obtain reads that failed to align against the host genome. These were then mapped against the genome of *L. braziliensis* MHOM/BR75/M2904 obtained from TriTrypDB v. 45 (<https://tritrypdb.org/tritrypdb/>) (Aslett et al. 2010). FeatureCounts v. 1.6.3 (Liao et al. 2014) was used to produce a count table summarizing gene-level abundance for each sample, and the corresponding annotation of the *L. braziliensis* genome was obtained in GFF format using TriTrypDB.

To perform digital cytometry, which allowed the estimation of immune cellular fractions from the RNA-seq data of biopsies, quantIseq was employed (Finotello et al. 2019). This method, specifically developed for RNA-seq data, performs deconvolution of cell fractions based on constrained least squares regression, yielding estimates for 10 immune cell

populations. The quantIseq bash script was run using default parameters and boxplots were produced to visually compare cell population estimates across samples. Due to multicollinearity in the expression of related cell types, such as Tregs and non-regulatory CD4⁺ cells, that hampers their deconvolution (Finotello et al. 2019), we obtained estimates of total CD4⁺ cells (including regulatory CD4⁺ cells) by summing the columns T.regs and T.cells.CD4 in the quantIseq output table.

All visualizations were produced in R 3.4.4 (<http://www.r-project.org/>) using base functions and the packages ggplot2 and circlize (Gu et al. 2014; Wickham 2010).

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