Genomic Profiling of Human *Leishmania braziliensis* Lesions Identifies Transcriptional Modules Associated with Cutaneous Immunopathology

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The host immune response has a critical role not only in protection from human leishmaniasis but also in promoting disease severity. Although candidate gene approaches in mouse models of leishmaniasis have been extremely informative, a global understanding of the immune pathways active in lesions from human patients is lacking. To address this issue, genome-wide transcriptional profiling of *Leishmania braziliensis*-infected cutaneous lesions and normal skin controls was carried out. A signature of the *L. braziliensis* skin lesion was defined, which includes over 2,000 differentially regulated genes. Pathway-level analysis of this transcriptional response revealed key biological pathways present in cutaneous lesions, generating a testable ‘metapathway’ model of immunopathology and providing new insights for treatment of human leishmaniasis.

**INTRODUCTION**

*Leishmania braziliensis* has a spectrum of clinical manifestations, all of which are associated with immunopathology (de liveira and Brodskyn, 2012). Patients develop small nodules at the site of infection that progress to chronic ulcerated lesions. We hypothesize that, although parasite infection acts as an initial trigger for lesion development, it is the immunopathologic response that determines disease severity. Thus, defining the host inflammatory pathways within leishmania lesions is crucial for the development of new treatment modalities.

Many studies have examined the systemic immune response in *L. braziliensis*-infected patients, and show that cells from patients release pro-inflammatory molecules in response to leishmania antigen (Bottrel et al., 2001; Follador et al., 2002; Vargas-Inchaustegui et al., 2010). These responses likely contribute to both the control of the parasites and the pathologic inflammatory response in the lesions (Bosque et al., 1998; Bacellar et al., 2002; de Oliveira and Brodskyn, 2012; Giudice et al., 2012). Although important, these systemic responses may not reflect what is occurring at the site of infection. Indeed, recent studies of lesion biopsies from *L. braziliensis* patients have revealed an unexpected pathologic role for CD8 T cells during disease, which would not have been obvious from studies on systemic responses (Novais et al., 2013; Santos Cda et al., 2013).

Transcriptome analysis has helped elucidate critical genes expressed during interactions between leishmania parasites and human macrophages (Ramirez et al., 2012). In addition, a genomic profiling has been reported for leishmania lesions from patients, in which the authors compared cutaneous leishmaniasis (CL) and mucosal leishmaniasis (Maretti-Mira et al., 2012). To our knowledge, however, ours is the first report to dissect the changes that occur in the skin after infection with leishmania when compared with normal skin. Using a genome-wide transcriptional analysis, we report on the pathways present in *L. braziliensis* lesions and propose a hypothetical ‘metapathway’ of immunopathology that drives disease.

**RESULTS**

**Comparative transcriptomics of *L. braziliensis* lesions and normal skin**

We performed genome-wide transcriptional profiling on 25 biopsies from *L. braziliensis* patients (Supplementary Table S1 online) and 10 normal skin biopsies obtained from non-endemic controls. Principal component analysis (PCA) of the entire data set showed that principal component 1 (PC1) accounted for 54.3% of the variation in the data and resolved samples into two main groups, normal and lesion skin. PC2 accounted for a smaller amount of variation (12.4%) occurring
within both these groups (Figure 1a). The separation of lesion and control samples along a single principal component indicated that differentially expressed genes could be identified with high statistical confidence.

Analysis of L. braziliensis lesions compared with normal skin identified 2,028 differentially expressed genes (≥2-fold, false discovery rate ≤1%) (Figure 1b). Hierarchical clustering (HC) based on Pearson’s correlation delineated two major clusters. Cluster 1 comprises 947 genes whose abundance is decreased in lesions, relative to normal skin. The 10 most “repressed” genes from this cluster include genes associated with maintenance of skin barrier function, such as keratin-27 (KRT27), filaggrin-2 (FLG2), and dermcidin (DCD) (Figure 1c). Cluster 2 comprises 1,081 transcripts that were more abundant in lesions compared with normal skin. The most strongly ‘induced’ members from this cluster included genes associated with inflammatory cell recruitment (CXCL9, CXCL10, and CCL8) and cytotoxicity (GZMA, GZMB, and GLYN) (Figure 1d).

Functional enrichment and pathway analysis of the L. braziliensis lesion

We next carried out a functional enrichment analysis using Gene Ontology (GO) terms (Ashburner et al., 2000). Genes upregulated in lesions were enriched in GO terms related to inflammation, host defense, and chemotaxis (Figure 2a). In contrast, genes downregulated in lesions were associated primarily with fatty acid metabolism and epidermal development (Figure 2a). This enrichment analysis suggests that lesion development is associated with a remodeling of the local skin environment, marked by induction of a potent pro-inflammatory signature and a concomitant loss of epidermal and fatty acid metabolic signatures. Although useful for identifying general functional categories, GO enrichment analysis is biased in that it requires a relatively arbitrary selection of differentially expressed genes as input. Therefore, using gene set enrichment analysis (GSEA) analysis we leveraged manually curated pathway databases, including Reactome, Kyoto Encyclopedia of Genes and Genomes, Biocarta, and the Pathway Interaction Database (Nishimura, 2001; Vastrik et al., 2007; Schaefer et al., 2009; Kanehisa et al., 2014), to identify the key pathways enriched in lesions. Despite our finding that over 2,000 genes were differentially regulated in the L. braziliensis lesion, pathway analysis showed that much of this transcriptional response could be explained by a small number of pathways (Figure 2b). GSEA results confirmed a potent repression of fatty acid metabolism in the L. braziliensis lesion. To further investigate this altered metabolic profile, we

![Figure 1. Defining the transcriptome of the human L. braziliensis skin lesion.](https://www.jidonline.org)
Genomic Profiling of L. braziliensis Lesions

Identification of unique and conserved pathways associated with skin lesion disease

Our data identified core pathways associated with the L. braziliensis lesion; however, it remained an open question as to whether they were a common feature of skin inflammation. To address this question, we compared our data with those of lesions from human psoriasis lesions (Figure 3). As expected, only the L. braziliensis lesion was enriched for "JAK/signal transducer and activator of transcription signaling", the "IFN-γ pathway", and the "Leishmania" Kyoto Encyclopedia of Genes and Genomes pathway, all of which include genes well known to be critical mediators of protection from this parasite. In addition, L. braziliensis lesions were uniquely enriched for "NK-mediated cytotoxicity" and "allograft rejection", whereas our analysis showed that the "graft versus host disease" is enriched in both diseases. However, we found it to be much more strongly enriched in L. braziliensis lesions, suggesting that this pathologic response is a dominant feature of CL. Similarly, this analysis also identified inflammasome activation as a major pathway activated in L. braziliensis lesions but not in psoriasis. Several pathways were preferentially enriched in psoriasis and primarily included cell proliferation and nucleotide metabolism. Finally, several pathways were enriched in both diseases, including IFN-α/β signaling, nucleotide-binding and oligomerization domain-like receptor signaling, cytokolic DNA sensing, defensins, and regulation of apoptosis. Taken together, this comparison indicates that L. braziliensis induces a molecular signature of disease distinct from psoriasis.

Early- and late-stage lesions are transcriptionally indistinguishable

Lesions from L. braziliensis patients could be classified into two categories based on the clinical stage of the disease, termed early and late (Figure 4 and Supplementary Table S1).

Figure 2. Functional enrichment analysis of L. braziliensis-infected skin. (a) GO enrichment analysis showing Biological Process terms enriched in induced genes from Figure 1b (red) or repressed genes (blue) in lesions relative to normal skin. (b) GSEA showing enriched pathways from the MSigDB C2 canonical pathway collection. Color-coded circles to the right indicate pathway database provenance. Columns represent samples and rows represent individual pathways, colored to indicate expression levels based on a Z-score. GO, Gene Ontology; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genome; MSigDB, Molecular Signatures Database; PID, Pathway Interaction Database.

Examined all genes known to be involved in either cholesterol or triglyceride and free fatty acid metabolism (Supplementary Figure S1 online). Interestingly, we identified a global repression of both cholesterol and free fatty acid biosynthesis (Supplementary Figure S1c—d online), and a significant increase in expression of lipid exporters (Supplementary Figure S1e—f online), suggesting that L. braziliensis lesions are characterized by dysregulated lipid biosynthesis. In contrast, lesions showed marked induction of at least five key pathways. As expected, cytokotoxicity and pathways involved in the generation of reactive oxygen species were strongly induced in the L. braziliensis lesion (Novais et al., 2013, 2014). In addition, this analysis identified at least three other pathways associated with the lesion transcriptome: (1) antigen processing and immunoproteasome activation; (2) nucleic acid sensing; and (3) inflammasome activation and apoptosis.
online). Patients with early lesions had a small papule with no evident ulceration, a median lesion size of 38 mm² (Figure 4a), and an illness duration of ≤30 days (Figure 4b). In contrast, patients with late lesions had an illness duration of ≥30 days, with ulcerated lesions with a median size of 250 mm². Despite these marked differences, PCA of the entire transcriptome (data not shown) and an HC of the differentially expressed genes failed to resolve early- and late-stage lesions as transcriptionally distinct disease states (Figure 4c). In addition, our analysis failed to find any significant differentially expressed genes between the two lesion stages, even when less stringent cutoffs were used (fold change ≥1.5 and \( P \leq 0.05 \)) (data not shown). The observation that \( L. \) braziliensis lesions at different clinical stages are indistinguishable by gene expression and pathway analysis (data not shown) reveals that the key pathways associated with \( L. \) braziliensis lesions are evident well before the development of ulcerated skin lesions, and therefore may be promoting cutaneous pathology, rather than simply arising as a consequence of disease.

**Identification of genes associated with a molecular signature of skin pathology**

We next sought to identify gene signatures that contributed to patient-to-patient variability in the lesion transcriptome. A PCA was carried out using only the 2,028 differentially expressed genes from lesion samples. PC1 accounted for 35.2% of the variability within the group of lesion samples, followed by 11.1% in PC2 (Figure 5a), and PC1 and PC2 together explained almost half of the variation between patients’ samples. This analysis showed that patients varied in their induction of this transcriptional program, but this variation was independent of age, sex, drug sensitivity (data not shown), and lesion stage (Figure 5a). To determine which genes had the strongest influence on these two principal components, and therefore contributed the most to variability in the lesion transcriptome between patients, we plotted the PCA ‘scores’ from all differentially expressed genes for PC1 and PC2 (Supplementary Figure S2 online). This analysis identified a subset of immune and skin barrier function genes, whose expression is variable across the patients. A subset of immune genes and skin barrier genes (Supplementary Figure S2 online) from our PCA score plot was selected for correlation analysis (Figure 5b). As expected, there was a strong positive correlation between functionally related genes (Figure 5b), such as components of the cytolytic granule (GZMB, GNLY, and PRF1) (Figure 5b), meaning that patients with high levels of granzyme B transcript in the lesion often had high levels of granulysin and perforin. In contrast, the
Activation of CD8 T cells requires recognition of antigens present in the skin of patients with cutaneous leishmaniasis (CL). We found that the expression of CD8 T cells is increased in CL lesions, and we propose that excessive expression of these chemokines brings more CD8 T cells to the skin, thereby exacerbating immunopathology.

Genes associated with the development and function of T helper type 1 (Th1) responses were highly expressed in L. braziliensis lesions, whereas genes associated with Th2 (Novais et al., 2014) or Th17 responses (data not shown) were not induced. This contrasts with the observations that Th2 and Th17 responses are induced in mucosal disease (Boaventura et al., 2010; Maretti-Mira et al., 2012). Our results are consistent with the strong Th1 response observed systemically in CL patients (Carvalho et al., 2012). Several genes downstream of IFN-γ were upregulated and may contribute to pathology. These data show an increased expression of immunoproteasome genes in CL, which helps in generating major histocompatibility complex class I epitopes from the parasite and ultimately increase CD8 T–cell activation. Also, studies indicate that the immunoproteasome contributes to inflammation (Muchamuel et al., 2009) and CD8 T–cell survival (Moberg et al., 2010). In addition to immunoproteasome-related genes, IFN-γ also induces expression of CXCL10 and CXCL9, both of which recruit activated T cells and NK cells (Dufour et al., 2002). Therefore, we propose that, in addition to its well-known function in parasite control (Kay and Scott, 2011), IFN-γ participates indirectly in immunopathological responses in L. braziliensis infection by inducing the recruitment of CD8 T cells and NK cells to the skin and triggering cytotoxicity by stimulating the immunoproteasome activation and antigen presentation to CD8 T cells.

We found that cytotoxicity is one of the main signatures of disease induced by L. braziliensis, a finding consistent with a previous study performed with a smaller number of samples (Novais et al., 2013). Although we find Th1 responses induced in lesions, the dominance of the cytolytic pathway is evident when one compares the fold change in IFNG and GZMB expression between normal skin and leishmanial lesions. As expected, IFNG is increased in expression (8.8 fold change) (Novais et al., 2014), but GZMB has a significantly higher fold change (50.9) (Novais et al., 2013). In L. braziliensis patients’ lesions, CD4 but not CD8 T cells produce IFN-γ, and thus the main function of CD8 T cells in the lesions of patients appears to be cytotoxicity (Santos Cda et al., 2013). We found that cytotoxic CD8 T cells mediated immunopathology in mice, but the mechanism by which cytotoxicity enhanced disease was unclear (Novais et al., 2013). In light of our transcriptome
analysis, we now hypothesize that the increased pathology mediated by CD8 T cells is due to activation of the inflammasome by release of DAMPs.

Activation of the inflammasome generates mature IL-1β, which promotes increased inflammation by stimulating the production of chemokines, such as IL-8, and also matrix metalloproteinases, which degrade the extracellular matrix leading to more damage to the skin. Our study indicates that genes associated with the inflammasome pathway (such as IL1B, AIM2, NLRP3, CASP1, and CASP5) are highly expressed in L. braziliensis lesions, suggesting that there is inflammasome activation and secretion of IL-1β during disease. In fact, ex vivo-cultured human L. braziliensis lesions release IL-1β protein into culture supernatants (Carvalho et al., unpublished data). However, the role that the inflammasome and subsequent IL-1β have in human disease is still unclear. IL-1β mRNA was previously found in lesions from L. braziliensis patients (Pirmez et al., 1993), and in individuals infected with L. mexicana IL-1β production has been linked to disease severity (Fernandez-Figueroa et al., 2012). Here, we expand those results by demonstrating that genes associated with two

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**Figure 5. Cytotoxicity and inflammasome-related genes inversely correlate with expression of skin barrier function genes.** (a) Principal component analysis of differentially expressed genes from L. braziliensis-infected patients. (b) Correlation heatmap showing selected modules of genes. Columns and rows represent individual genes, colored to indicate the correlation coefficient (r). (c–e) Log2 expression of (c) filaggrin and granulysin, (d) loricrin and interleukin-1β, and (e) desmocollin-1 and neutrophil cytosolic factor-1 in L. braziliensis-infected patients.

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**Figure 6. A putative “metapathway” driving immunopathology in cutaneous leishmaniasis.** Transcriptional modules (pathways) induced during leishmaniasis (shown on the left), with examples of genes that are differentially expressed in lesions (shown in the center), suggest a testable model of immune responses (shown on the right) that lead to immunopathology within leishmanial lesions, in which cytotoxicity has a central role.
inflammasome pathways, AIM2 and NLRP3, are upregulated in lesions and thus may have a previously unappreciated role in L. braziliensis human disease.

Skin diseases can share some characteristics. For example, dysbiosis of the skin has recently been considered a distinctive feature of both CL and psoriasis (Cho and Blaser, 2012; Naik et al., 2012). In addition, IFN-γ has been associated with immunopathology in both diseases, although by different mechanisms. In L. braziliensis infection, IFN-γ is thought to induce immunopathology by activating innate cells. In psoriasis, IFN-γ synergizes with other pro-inflammatory cytokines, notably IL-17, and induces activation of keratinocytes. Although Th17 responses have been implicated in L. braziliensis infection in mucosal leishmaniasis (Boaventura et al., 2010; Maretti-Mira et al., 2012), we could not detect differences in IL-17 transcripts in L. braziliensis patients, suggesting that, unlike psoriasis, L. braziliensis CL is not associated with a Th17 response. Our comparison of pathways enriched in these two diseases revealed additional differences. For example, although cytotoxicity has been implicated in both leishmaniasis (Novais et al., 2013) and psoriasis (Yawalkar et al., 2001; Prpic Massari et al., 2007), our data show that cytotoxicity is a more pronounced signature in L. braziliensis infection.

A surprising finding of our study was that the transcriptional profile of non-ulcerated lesions was similar to those of patients with ulcerated lesion. This result suggests that, early after infection, inflammatory pathways are activated in the skin, which may explain why lesions often develop despite early detection and treatment (Machado et al., 2002). Although our data are based on a fraction of the total lesion, as biopsies were collected from the border of the ulcer, we believe the results appropriately reflect the ongoing immune response as the ulcer is mainly composed of dead cells. As disease signatures are present before the ulcer develops, our data position cytotoxicity, immunoproteasome, and inflammasome as potential causes of lesion development, rather than as simply arising as consequence of disease.

Therapies that target the inflammatory response, without affecting mechanisms that kill the parasites, would be an ideal adjunct to drug treatment in leishmaniasis. Here, we have identified a hypothetical metapathway that leads from CD8 T-cell activation and cytolysis to IL-1β production. As cytotoxicity does not control L. braziliensis parasites (Novais et al., 2013; Santos Cda et al., 2013), nor does IL-1β appear to be protective in humans (Fernandez-Figueroa et al., 2012), blocking the major components of this metapathway should limit pathology without affecting parasite control.

**Materials and Methods**

**Ethics statement**

This study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines, and this study was approved by the Ethical Committee of the Federal University of Bahia (Salvador, Bahia, Brazil)(010/10) and the University of Pennsylvania IRB (Philadelphia, PA) (813390). All patients provided written informed consent for the collection of samples and subsequent analysis.

**Patients and Biopsies**

All CL patients were seen at the health post in Corte de Pedra, Bahia, Brazil, which is a well-known area of L. braziliensis transmission. The criteria for diagnosis were a clinical picture characteristic of CL in conjunction with parasite detection or a positive delayed-type hypersensitivity response to leishmania antigen. Prior to therapy, biopsies were collected at the border of the lesions using a 4-mm punch before therapy. Normal skin samples were taken from volunteers who were living in a non-endemic area without a history of leishmaniasis.

**Transcriptional Profiling and Functional Enrichment Analysis**

Microarrays and data analyses were carried out as previously described (Beiting et al., 2014). Briefly, Illumina HumanHT-12 version-4 beadchips (Illumina, San Diego, CA) were hybridized with biotin-labeled cRNA generated from 10 normal skin, 8 early, and 17 late lesion samples. Data analyses were carried out using the statistical computing environment, R (v3.0.2), the Bioconductor suite of packages for R, and RStudio (v0.97; Boston, MA). Probesets that were differentially regulated ≥2-fold (false discovery rate ≤1%), after controlling for multiple testing using the Bonferroni–Hochberg method (Reiner et al., 2003), were used for HC and heatmap generation. Data have been deposited on the GEO database for public access (GSE# GSE55664). GSEA (Mootha et al., 2003; Subramanian et al., 2005) was carried out using the Broad Institute’s MSigDB (v4.0) and either the GSVA bioconductor package (Figure 2) (Hanzelmann et al., 2013) or the GSEA preranked tool (Figure 3) to query the “C2: Canonical Pathways” collection in the MSigDB, which consists of 1,310 gene sets, or “signatures”, representing annotated pathways.

**Comparison of L. braziliensis and Psoriasis Lesion Transcriptomes**

L. braziliensis data were compared with the MAD-3 human psoriasis data set (Tian et al., 2012), a meta-analysis of three independent psoriasis gene expression studies including 334 paired samples (lesion and non-lesion biopsies) from 167 patients (Yao et al., 2008; Gudjonsson et al., 2009; Suarez-Farinas et al., 2012). The MAD-3 data set was first filtered to remove nonspecific Affymetrix probesets (probeset identifiers ending in “.X_at”). Genes with multiple probesets were used to calculate a mean fold change relative to non-lesion controls. A total of 17,061 genes in common between the psoriasis MAD-3 data set and our L. braziliensis lesion data were used for carrying out a competitive GSEA analysis. Both data sets were rank ordered by Log2 fold change in expression between lesion and control and used as input for the GSEA preranked algorithm (Mootha et al., 2003; Subramanian et al., 2005). GSEA results were explored using the network analysis software Cytoscape (Cline et al., 2007; Smoot et al., 2011) and the Enrichment Map plugin (Merico et al., 2010), in order to identify common and unique pathways.

**Conflict of Interest**

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/ijid

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