Published in final edited form as:

Sci Transl Med. 2019 November 20; 11(519): . doi:10.1126/scitranslmed.aax4204.

Variable gene expression and parasite load predict treatment outcome in cutaneous leishmaniasis

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Abstract

Patients infected with *Leishmania braziliensis* develop chronic lesions that often fail to respond to treatment with anti-parasite drugs. To determine whether genes whose expression is highly variable in lesions between patients might influence disease outcome, we obtained biopsies of lesions from patients prior to treatment with pentavalent antimony, performed transcriptomic profiling, and identified highly variable genes whose expression correlated with treatment outcome. Amongst the most variable genes in all the patients were components of the cytolytic pathway, the expression of which correlated with parasite load in the skin. We demonstrated that treatment failure was linked to the cytolytic pathway activated during infection. Using a host-pathogen marker profile of as few as 3 genes, we showed that eventual treatment outcome could be predicted before the start of treatment in two separate patient cohorts (n=21 and 25 cutaneous leishmaniasis patients). These findings raise the possibility of point-of-care diagnostic screening to identify patients at high risk of treatment failure and provide a rationale for a precision medicine approach to drug selection in cutaneous leishmaniasis, and more broadly demonstrate the value of identifying genes of high variability in other diseases to better understand and predict diverse clinical outcomes.

One Sentence Summary:

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Author contributions: C.F.A., D.P.B. and P.S. conceptualized the study; C.F.A., B.N., A.M.M., and D.P.B.; participated in the methods and validated the results; C.F.A. analyzed the results; C.F.A., F.O.N., B.N., A.M.M., L.P.C., E.M.C., D.P.B. and P.S. participated in the investigation; D.P.B., L.P.C., E.M.C., and P.S. provided resources and funding acquisition; C.F.A., D.P.B. and P.S. participated in data curation; C.F.A., D.P.B. and P.S. contributed in the writing of original draft, review and editing; C.F.A., F.O.N., B.N., A.M.M., L.P.C., E.M.C., D.P.B., and P.S.; C.F.A., D.P.B. and P.S. supervised this study.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: Raw sequence data are available at on the Sequence Read Archive (SRA; project # PRJNA525604), and tabular count data are available on the Gene Expression Omnibus (GEO, accession # GSE127831). All code used to analyze RNA-seq data and clinical metadata from this study is available on Github and has been archived on Zenodo (DOI: [10.5281/zenodo.3374884](https://doi.org/10.5281/zenodo.3374884)). This repository contains quality control analysis of raw data, as well as an RMarkdown document that integrates code with outputs and which was used to generate data file S2.

Variability in immune gene expression predicts treatment outcome in cutaneous leishmaniasis.

Introduction

Cutaneous leishmaniasis (CL) is characterized by the development of cutaneous lesions that may resolve in several months or in some cases lead to the development of metastatic lesions (1, 2). The response to drug treatment is highly variable, and antimony – the current first-line treatment for cutaneous leishmaniasis in Brazil – is not only toxic but also has a high failure rate (1–6). As the current drug therapy for leishmaniasis, pentavalent antimony, is often unsuccessful, defining the pathways leading to disease could potentially identify targets for host-directed therapies. Studies with patients combined with parallel studies in experimental murine models have revealed that tissue destruction in CL is initiated by cytolytic CD8⁺ T cells that lead to inflammasome activation and consequent IL-1 β production (7–13). IL-1 β then promotes increased inflammation, enhancing disease severity. Blocking either Nlrp3 or IL-1 β protects mice against CD8⁺ T cell-mediated immunopathology (8), indicating that these proteins might be important targets in host-directed therapies for the disease. However, despite overwhelming direct evidence that this cytolytic pathway promotes increased disease in experimental models (7, 8, 14–21) and indirect evidence in patients (8, 10–12, 22), a direct demonstration that this pathway influences disease outcome in patients is lacking.

In the past decade, advanced sequencing platforms have accelerated the identification of both host and pathogen gene expression signatures associated with immunopathogenesis, clinical progression, and response to treatment of infectious diseases (23–27). We predicted that an assessment of genes that are differentially expressed between infected patients might identify genes associated with disease outcome that could be useful as targets for therapeutic design or as predictors of treatment failure. Given that cytolytic genes are induced early in CL lesions (9), we specifically hypothesized that variations in the magnitude of expression of such genes might influence disease outcome and provide the potential markers to identify patients who may fail conventional therapy.

To address this hypothesis, we performed RNA sequencing on lesion biopsies taken from CL patients at the initiation of treatment with pentavalent antimony and identified highly variable genes upregulated relative to healthy skin. Following RNA-sequencing (RNA-seq) of biopsies of two distinct patient cohorts and statistical filter strategies, we focused on a set of genes that were upregulated in lesions from patients who failed treatment, which included genes involved in cytolysis. Using dual RNA-seq and qPCR to identify both parasite and host transcripts, we discovered that small differences in the parasite load correlated with cytolytic gene expression as well as treatment outcome, suggesting that parasites were driving this cytolytic pathway. Taken together, these studies demonstrate that differences in the expression of the cytolytic pathway leading to IL-1 β production influence disease outcome, and that variability in the expression of a small number of genes, as well as parasite load, can predict responsiveness to treatment.

Results

Transcriptional profiling indicates that cytotoxicity-related pathways are enriched in *L. braziliensis* lesions

To evaluate the transcriptional signatures associated with disease, we carried out RNA sequencing on skin biopsies collected from 21 *L. braziliensis* patients before pentavalent antimony treatment, and 7 uninfected endemic controls. Transcriptional profiles from lesions were distinguishable from healthy skin by principle component analysis (Fig. 1A), with 4255 genes identified as differentially expressed between the two groups (Fig. 1B). Confirming previous findings (9), we identified two cytotoxicity-related pathways included in the top 10 enriched pathways in lesions by gene set enrichment analysis (GSEA) (Fig. 1C) and found that many genes associated with cytolysis, including *GZMB*, *PRF1*, *GNLY*, and *IL1B* exhibited significant enrichment over healthy controls (false discovery rate [FDR] < 0.01) (Fig. 2A). Genes associated with B cell activation, phagocytosis, complement activation, and Fc gamma receptor signaling were amongst the most highly expressed genes in lesions (28) (fig. S1).

Highly variable genes are present in lesions from *L. braziliensis* patients

We previously reported that patient-to-patient variability in gene expression during CL could not be explained by the stage of disease (9). As cytolysis promotes pathology through the release of IL-1 β (8), we hypothesized that variability in the expression of genes central to these pathways may impact clinical outcome in patients. Indeed, we found that genes associated with cytotoxicity (*GZMB*, *PRF1* and *GNLY*) and *IL1B*, all of which promote pathology in CL, varied substantially between patients (Fig. 2A). To explore the variability of gene expression more broadly in patients with CL, we calculated the coefficient of variation (cV) for all 2037 genes upregulated in lesions (Fig. 2B). We defined the top 250 most variable genes ($cV \geq 0.65$) as ‘Variable Transcripts Associated with Lesions’ (ViTALs). Gene ontology analysis of ViTALs identified an enrichment of genes involved in ‘B cell responses’ (Cluster 1, yellow) and ‘cell chemotaxis signaling-related’ genes (Cluster 2, blue), FDR<0.05 (data file S1). *GZMB*, *PRF1*, *GNLY* and *IL1B* (Cluster 3, red) also exhibited highly variable expression between patients (Fig. 2B). In contrast, genes that were induced in lesions but which showed low variability amongst patients ($cV < 0.35$) were enriched for type I and II interferon signaling, antigen processing and presentation, and histone-related gene expression, suggesting that induction of these pathways is common to all lesions but is not associated with clinical outcome.

Variability in cytotoxicity-related gene expression is associated with treatment outcome

Because biopsies were collected prior to treatment, we could only stratify the samples after monitoring the patient’s treatment outcome. 14 individuals had lesions that resolved within 90 days of treatment, whereas the lesions of 7 individuals failed to resolve in this time period and required additional rounds of chemotherapy; these patient groups are hereafter referred to as ‘cured’ and ‘failed’, respectively (Fig. 3A). None of the 250 ViTALs were associated with patient age, delayed-type hypersensitivity response, or lesion size (Spearman correlation test, FDR>0.05), nor did these clinical covariates show any correlation with sex or treatment outcome (Mann-Whitney U test, $P > 0.05$), (fig. S2). In contrast, 31 out of 250

ViTALs (12.4%) were differentially expressed between patients who failed treatment and those that cured after the first round of pentavalent antimony treatment (Fig 3B). To determine if any of these genes also correlated with treatment failure in a second data set, we performed the same analysis on a cohort that we previously studied (referred to hereafter as “2016 dataset”) (28). Eight of the 31 ViTALs significantly correlated with treatment failure in both the current dataset and the 2016 dataset ($\log_2$ fold change ($\log_2FC$) ≥ 1 and P value ≤ 0.05). These included key components of the cytolytic machinery (*GZMB*, *PRF1* & *GNLY*); genes associated with cytolytic T cells (*UNC13A*, *APOBEC3A*, & *KIR2DL4*) (29–32); *CCL4*, which binds to *CCR5* and is expressed on CD8+ T cells; and IFN- γ . Consistent with these results, Gene Ontology (GO) enrichment analysis carried out on all 250 ViTALs in our current dataset showed significant enrichment of several pathways, including ‘Graft-versus-host-disease’ and ‘Natural killer cell mediated cytotoxicity’ ($FDR < 0.0001$). Similarly, GO analysis of 595 ViTALs identified in the 2016 dataset ($cV < 0.19$) confirmed enrichment of the same pathways ($FDR < 0.02$). Taken together, these data identify a key set of transcripts and pathways whose expression is associated with treatment failure across multiple studies. We used CombiROC analysis (33) to determine the number of markers needed to predict pentavalent antimony treatment outcome and found in the current cohort of patients that a combination of 3 genes, *GNLY*, *IL1B* and *IFNG*, produced an area under the curve (AUC) of 0.86–0.96, indicating that these genes predict treatment outcome in this group with 86–96% accuracy (fig. S3).

Variable expression of cytotoxic genes is associated with increased abundance of CD8+ effector T cells and NK cells in lesions of patients who fail treatment

Variability in expression of cytotoxic genes could be explained by either a difference in the per cell expression of these genes between patients, or differences in the numbers of cytotoxic cells present in lesions from different patients. To distinguish between these two possibilities we used ImmQuant (34) to infer the cell type composition of lesions using cell-type-specific marker genes present in our RNA-Seq data (Fig. 4). ImmQuant analysis identified high variability in the predicted relative abundance of CD8+ T cells and NK cells between patients with CL (Fig. 4A). Furthermore, patients who failed treatment exhibited elevated proportions of CD8+ T cells and NK cells, but not CD4+ cells (Fig. 4B). There was a positive correlation between the expression of ViTAL cytotoxic genes (*GZMB*, *PRF1* and *GNLY*) and the predicted relative abundance of CD8+ T cells and NK cells ($\rho > 0.6$ and $P < 0.002$) (Fig. 4C). These results suggest that the increase in cytolytic genes is likely due to increased recruitment of cytotoxic cells to the lesion. To confirm this conclusion, we assessed the percentage of GzmB-expressing CD8+ T cells in patient lesions prior to treatment using flow cytometry, and observed a significant positive correlation between the expression of CD8+ T cells and GzmB ($\rho = 0.75$, $P = 0.003$) (Fig. 4D). With one exception, the patients who failed treatment had the highest percentage of GzmB+ CD8+ T cells in their lesions (Fig. 4D), which was consistent with our RNA-seq results. *CCL3* and *CCL4* were upregulated in lesions from patients who failed treatment, suggesting these genes might be responsible for increased recruitment of cytotoxic cells to the site of the infection. Consistent with this notion, we observed a positive Spearman’s correlation between the expression of *CCL3*, *CCL4*, and *CCR5* (the receptor for *CCL3* and *CCL4*) and the predicted relative abundance of CD8+ T cells and NK cells ($\rho > 0.6$ and $P < 0.003$) (Fig. 4C). These

findings reinforce the role of CD8⁺ T cells as mediators of pathology during CL infection and implicate the recruitment of cytolytic CD8⁺ T cells in treatment failure.

Parasite load correlates with gene expression variability

Lesions from patients infected with *L. braziliensis* have been reported to contain very few parasites (35) and consistent with this, we previously reported that *L. braziliensis* transcripts could only be identified in a subset of patients by RNA-seq (28). Similarly, in this study we detected parasite transcripts in only 13 out of 21 samples by dual RNA-seq of host and parasite transcripts (Fig. 5A). To better quantify the parasites in lesions we used qPCR (fig. S4) and found that 19 of the 21 lesion biopsies were positive by qPCR for *L. braziliensis* 18S rRNA (Fig. 5B). We found a strong positive correlation between the abundance of parasite transcripts detected by RNA-seq and absolute number of parasites determined by qPCR ($\rho=0.90$ and $P<0.0001$) (Fig. 5C). We next asked if parasite load was associated with the cytotoxic signature seen in lesions from patients who failed treatment, and found a strong correlation between parasite number measured by either qPCR or RNA-seq, and both the expression of ViTALs (Fig. 5D and fig. S5) as well as the predicted relative abundance of CD8⁺ T cells and NK cells by ImmQuant (Fig. 5E). Highly expressed genes with limited variability between patients ($cV<0.65$), such as *MMP2*, *STAT1*, *CXCL10* and *CD19*, did not correlate with parasite load ($\rho<0.29$ and $P>0.23$) (fig. S6). These results suggest that parasite load contributes to the increased recruitment of cytotoxic cells to the site of infection and, consequently, the high abundance of transcripts from cytotoxic genes detected in lesions.

Parasite load predicts treatment outcome

Given the association between ViTALs and treatment failure and ViTALs and parasite load, we hypothesized that parasite load would predict treatment failure. We found that lesions from patients who failed the first round of treatment had more parasites compared to the lesions from patients who were cured, as measured by qPCR (median of 34,370 parasites versus 17,805 parasites, respectively; $P<0.001$) (Fig. 6A), or by RNA-seq (median of 5,198 CPM vs. 78 CPM, $P<0.0001$) (Fig. 6B, left). We confirmed these results by applying the same analytic pipeline used in the current study to the 2016 RNA-seq dataset (Fig. 6B, right) (28). Finally, Kaplan-Meier analysis revealed that patients with biopsies containing more than 32,000 parasites per 4 mm biopsy took nearly two months longer to heal than those with less than 32,000 parasites ($P=0.01$) (Fig. 6C). ROC analysis showed that parasite load in lesions from CL lesions by itself predicts treatment outcome with 96% accuracy (Fig. S3). These data suggest that the parasite load may have a direct and proportional effect on the recruitment of cytotoxic cells, thereby affecting how patients respond to treatment.

Discussion

Patients infected by *L. braziliensis* develop chronic ulcerated lesions which often fail to respond to drug treatment (3). By analyzing gene expression in lesions from these patients we identified Variable Transcripts Associated with Lesions ('ViTALs') that correlated with treatment failure, including several components of the cytolytic machinery (*GZMB*, *PRF1*, *GNLY*). We and others previously found that cytolysis is a major component of cutaneous lesions (7–12), and from a combination of human and murine studies we discovered that

cytolysis leads to NLRP3 activation, IL-1 β production, and subsequent severe inflammation (8). We found that blocking components of this pathway, such as NLRP3 or IL-1 β , ameliorated severe disease in murine models (8). Although these studies implicated cytolysis and IL-1 β production in the severity of cutaneous leishmaniasis, here we directly demonstrated that disease outcome in patients, in this case as assessed by treatment failure, associates with the degree of ongoing cytolysis within lesions. Thus, these results substantially strengthen the idea that therapies directed at blocking the NLRP3 inflammasome and IL-1 β in combination with anti-parasite drugs might be advantageous for patients.

Transcriptome profiling in infectious diseases is a powerful approach to identify the pathways that lead to disease and can be used to understand the pathogenesis of CL (9, 28, 36). Here, we hypothesized that the genes whose expression was most variable between patients would be instrumental in dictating treatment outcome. Our results confirm our previous findings that B cell-associated transcripts are highly upregulated in the lesions of patients infected with *L. braziliensis* (28), and demonstrate their variable expression between patients. Why immunoglobulin transcripts are upregulated in patient lesions remains unclear, but might reflect a role in limiting immune responses, as IL-10 has been associated with parasite persistence (37) and IgG opsonized parasites induce IL-10 in macrophages (38).

We next looked for correlations between highly variable genes and clinical outcome, and by analyzing two independent sets of patient data we were able to identify 8 ViTALs that correlated with treatment failure in both cohorts. These included *PRF1*, *GNLY*, and *GZMB*, directly linking for the first time these three cytolytic genes with treatment outcome. We also identified two genes (*UNC13A*, *APOBEC3A*) that have not previously been associated with leishmaniasis, but are related to genes that may influence cytolysis. For example, *UNC13D* regulates cytolytic granule secretion (32), whereas *APOBEC3G* improves cytolytic T cell recognition of target cells (31). Another gene, *KIR2DL4*, is expressed on CD8+ T cells and NK cells, and thus 6 out of 8 of the genes that correlated with treatment failure appear to be related to cytolysis. In addition, we found that *CCL4* was the only chemokine that correlated with treatment failure. As *CCL4* binds to *CCR5* which is expressed on CD8+ T cells, expression of this chemokine gene may also relate to CD8+ T cell recruitment to the lesions. Finally, we also found that *IFNG* correlated with treatment failure, highlighting that treatment failure is not likely to be due to the absence of a protective immune response.

To determine if the number of parasites influenced variability in gene expression, we quantitated parasites per 4mm biopsy by qPCR and compared these values to RNA-seq reads mapped to the *L. braziliensis* transcriptome. Using ImmQuant to infer relative cell composition from our RNA-seq data, we found that lesions with a higher number of parasites also exhibited an increased abundance of CD8+ effector T cells and NK cells, as along with higher expression of *GZMB*, *PRF1*, *GNLY* and *IL1B*. Correspondingly, prior to treatment, the parasite load in the lesions from patients for whom treatment failed was higher than in patients for whom treatment was successful. Differences in parasite number could be due to differing host responses, although it did not appear to be due to a lack of *IFNG* gene expression. Alternatively, the difference in parasite number could also be due to variation in parasite strains or the inability of the drug to eliminate the larger parasite

burden. However, the parasite burden varied relatively little across the lesion samples examined in this study and we favor the alternative possibility that small differences in parasite burden maintain a positive inflammatory feedback loop (fig. S8). Alternatively, the inflammation promoted by increased cytotoxicity may lead to recruitment of more cells that can be infected, thus leading to parasite accumulation in the lesion. However, in previous experimental models where we observed CD8⁺ T cell-mediated cytotoxicity and increased disease, depletion of CD8⁺ T cells or blocking the inflammasome pathway limited disease without changing the parasite burden (21).

There are important limitations to this study, one of which was the inability to monitor gene expression or parasite burden over time. Such information would allow us to determine if there were differences between patients in the ability of pentavalent antimony treatment to kill parasites, and whether a reduction in cytolytic gene and IL-1 β expression during treatment was associated with treatment success. Another limitation is that these studies only considered *L. braziliensis* patients, and in patients infected with different leishmania species cytolysis may be less important in promoting pathology. Finally, while we have associated treatment failure with expression of several genes linked with cytolysis, our results do not show that cytolysis or the production of IL-1 β is actually inhibiting treatment success. Studies from our experimental models indicates that this is likely, but only a clinical trial blocking IL-1 β or the pathway leading to IL-1 β will determine if this is indeed what is happening in patients.

In addition to identifying pathways leading to disease in patients infected with *L. braziliensis*, this study identified potential markers of treatment failure, which could have a significant impact on patient treatment. Pentavalent antimony is the drug of choice in Brazil for CL and is administered by daily intravenous injection for 20 days. In addition to having toxic side effects, this drug often fails to resolve disease and those patients that fail the first round of treatment undergo a second or, if needed, a third round of treatment (4–6). Prospective identification of patients at high risk of failing antimony treatment would save patients from treatment with an ineffective drug, and would expedite implementation of alternative treatments, such as liposomal amphotericin, to those who would benefit most from them. It remains to be determined if expression of certain ViTAL genes will be useful in predicting treatment outcome for other anti-leishmania drugs. However, since our prediction is that parasites promote increased cytolysis and inflammation in turn leading to greater pathology, we predict that drugs that are more efficient at eliminating parasites might break the cycle of inflammation.

By exploiting RNA-seq data we have substantially expanded our understanding of the pathogenesis of CL. We found that genes that vary in expression between patients identify pathways that can influence treatment outcome. These results directly demonstrate the significance of cytolysis in *L. braziliensis* patients and link treatment outcome to higher expression of genes associated with cytolysis. These results also suggest that experimental murine infections that mimic the CD8⁺ T cell-mediated cytolysis seen in patients may be better models for testing drugs than infection of C57BL/6 mice with *Leishmania* (7, 8, 21). In addition to CL, there are several infectious diseases where CD8⁺ cytolytic T cells have been implicated in promoting increased pathology, including cerebral malaria (39, 40),

trypanosomiasis (41), and coxsackievirus (42), raising the possibility that variability in the expression of genes associated with cytolysis might predict disease progression in these diseases as well. Furthermore, we have identified several genes that had not previously been known to influence leishmaniasis, and studies are ongoing to evaluate their role in disease. Finally, this study identifies parasite load as a driving factor in cytolytic gene expression, and demonstrates that parasite load, as well as cytolytic gene expression, might have potential as markers of anti-leishmania drug treatment failure. Identification of markers of disease progression in leishmaniasis could facilitate a substantial improvement in patient care, and should spur on future studies to identify new markers of disease progression in other clinical forms of leishmaniasis, a critical need for this widespread but neglected tropical disease.

Materials and Methods

Study Design

Our goal was to determine if analysis of CL lesions prior to treatment with pentavalent antimony would implicate genes associated with treatment failure that would define mechanisms of pathogenesis and identify potential markers of treatment failure. A characteristic skin lesion positive PCR for *L. braziliensis* and a positive intradermal skin test with leishmania antigen were used to identify patients with CL. Exclusion criteria included previous anti-leishmanial treatment, individuals under 18 years old, pregnancy, or the presence of other comorbidities. Prior to treatment, a 4mm punch biopsy was collected from CL patients and endemic controls (table S1) in Corte de Pedra, Bahia, Brazil and either stored in RNeasy Lysis Buffer (Qiagen) or cells collected from lesions for flow cytometry. Patients were given standard of care treatment (daily intravenous injections of pentavalent antimony; 20mg/kg/day for 20 days). At day 90 after the start of treatment patients were evaluated for lesion resolution. Cure was defined as re-epithelialization of lesions and the resolution of inflamed borders. Patients with active lesions at 90 days were defined as failing treatment and were given an additional round of chemotherapy. This study was conducted according to the principles specified in the Declaration of Helsinki and under local ethical guidelines (Ethical Committee of the Maternidade Climerio de Oliveira, Salvador, Bahia, Brazil; and the University of Pennsylvania Institutional Review Board). Informed consent for the collection of samples and subsequent analysis was obtained.

Transcriptional profiling by RNAseq

Lesions were homogenized with a MP tissue homogenizer (MP Biomedicals) and RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to manufacturer instructions and used to prepare sequence-ready libraries using the Illumina TruSeq Total Transcriptome kit with RiboZero Gold rRNA depletion (Illumina). Quality assessment and quantification of RNA preparations and libraries were carried out using an Agilent TapeStation 4200 and Qubit 3, respectively. Samples were sequenced on an Illumina NextSeq 500 to produce 75 bp single end reads with a mean sequencing depth of 45 million reads per sample for healthy controls and 56 million reads per sample for CL patients. Raw reads from this study, or from a previously published study (28), were mapped to the human reference transcriptome (Ensembl; Homo sapiens version 86) using Kallisto, version 0.44.0 (43). For identification

of parasite transcripts, raw data was filtered to remove human reads using KneadData (44), and remaining reads were mapped to the *L. braziliensis* reference transcriptome, MHOM/BR/75/M2904 version 2 (Ensembl) (fig. S7). Raw sequence data is available on the Gene Expression Omnibus (GEO, accession # GSE127831).

All subsequent analyses were carried out using the statistical computing environment, R version 3.5.1 (45) in RStudio version 1.1.456 and Bioconductor, version 3.8 (46). Briefly, transcript quantification data was summarized to genes using the TxImport package (47) and normalized using the TMM method in EdgeR (48). Genes with < 1 count per million (CPM) in n+1 of the samples, where n is the size of the smallest group of replicates, were filtered out. Normalized, filtered data were variance-stabilized using the VROOM function in limma (49) and differentially expressed genes were identified with linear modeling using limma (FDR = 0.01; absolute logFC = 1), after correcting for multiple testing using Benjamini-Hochberg. GO analysis was carried out using DAVID (50) and biological process terms. GSEA was carried out using GSEA software version 3.0 and the 'C2' canonical pathways collection from MsigDB (50, 51). ImmQuant (34), together with the DMAP cell phenotyping database (52), were used to predict the relative abundance of different cell-types from RNA-seq data. CombiROC software was used to compute the best sensitivity and specificity for different gene combinations and plot the results in as ROC (33). Parasite load ROC analysis was performed using GraphPad Prism v7. The full RNAseq data analysis pipeline is documented in the supplementary code file (data file S2), and all code is available on Github and has been archived on Zenodo (DOI: [10.5281/zenodo.3374884](https://doi.org/10.5281/zenodo.3374884)).

Quantification of parasite burden in cutaneous leishmaniasis lesion biopsies by qPCR

The same lesion RNA preparations used for RNA-seq were used to quantify parasite burden by qPCR. A standard curve was prepared from total RNA extracted from 10^7 *L. braziliensis* promastigotes recovered from axenic culture using the RNeasy Plus Mini Kit (Qiagen), and cDNA was generated with the High Capacity RNA to cDNA kit (Applied Biosciences). qPCR was carried out on a ViiA7 machine (Applied Biosciences) using Power SYBR green master mix (Applied Biosciences) and primers targeting the *L. braziliensis* 18S ribosomal subunit (F: 5'-TACTGGGGCGTCAGAG-3' and R: 5'-GGGTGTCATCGTTTGC-3') (53, 54) and human *GAPDH* (housekeeping gene, F: 5'-GGTGTGAACCATGAGAAGTATGA-3' and R: 5'-GAGTCCTTCCACGATACCAAAG-3') (fig. S4). All reactions were carried out in duplicate and data is presented as parasites per 4mm biopsy.

Flow cytometry

An independent set of 13 biopsies were collected from patients with CL prior to treatment. Biopsies were treated with Liberase (Roche) for 90 mins at 37°C and 5% CO₂, dissociated and passed through a 50 µm Medicon filter (BD Pharmingen). Cells were surface- and intracellularly stained with anti-CD8b PeCy5.5 (eBioscience) and anti-granzyme B APC (Invitrogen), with data collected on a FacsCanto (BD) and analyzed by FlowJo software (Tree Star).

Statistical analysis

Coefficient of Variation (cV) was calculated using FinCal v0.6.3 (55). Differential gene expression analysis was performed with limma package (49). Permutational multivariate analysis of variance (PERMANOVA) was used to compare distances between gene expression profiles of CL lesions and healthy skin. Receiver operating characteristic (ROC) curves were made with plotROC package to calculate area under the curve (AUC) for potential markers of treatment outcome (56). Mann-Whitney U tests and Spearman's rho (ρ) correlations were performed in the R statistical environment or GraphPrism v7, and $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We thank Dr. Luiz Guimarães and Ednaldo Lago for assistance with patient screening and sample collection.

Funding: This work was supported by U01 AI088650 (International Collaborations in Infectious Disease Research, ICIDR) to P.S., P50 AI030639 (Tropical Medicine Research Center, TMRC) to E.M.C., and the Center for Host-Microbial Interactions (CHMI).

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Fig. 1. Cytotoxicity-related pathways are enriched in CL lesions relative to healthy skin. (A) Principal component analysis showing PC1 and PC2 for RNA-seq data from healthy subjects (n=7) and CL lesions (n=21) from this study. A PERMANOVA statistical test was used to compare the two clinical groups. (B) A volcano plot showing up-regulated (red; n=2037) and down-regulated genes (blue; n=2218) in biopsies from patients relative to healthy subjects (FDR<0.01 and logFC>1). (C) Gene Set Enrichment Analysis (GSEA) showing two cytotoxicity-related pathways included in the top 10 enriched pathways in lesions relative to healthy skin. HS, healthy skin; PC, principal component; DEG, differential gene expression; FC, fold change; NES, normalized enrichment score; FDR, false discovery rate; adj.P.value, adjusted P value.

Fig. 2. Identification of highly variable gene expression signatures in CL lesions.

(A) Box plots showing expression of *GZMB*, *PRF1*, *GNLY* and *IL1B* in HS (n=7, grey) and CL lesions (n=21, red). Fold change (FC) comparing the sample with the highest versus lowest expression for each of the four genes in CL lesions. (B) Coefficient of variation (cV) for the 2037 upregulated genes in CL lesions relative to healthy subjects (HS). Histograms at the top and right show scatter plot distributions. The dashed line indicates an arbitrary cutoff (cV=0.65) for the most variable genes defined here as ViTALs, with 250 ViTAL genes above the line. Colored points indicate statistically significant pathways (FDR<0.01) within ViTALs. *GZMB*, *PRF1*, *GNLY* and *IL1B* genes are labeled. Legends are indicated inside the plot identifying enriched pathways or clusters of ViTALs. CPM, counts per million; FC, fold change. ViTALs, ‘Variable Transcripts Associated with Lesions’.

Fig. 3. Expression of cytotoxicity-related genes is associated with treatment outcome.

(A) The chart defines the overall steps of study design from day 0 to day 90. A representative photograph of a lesion shows in a white circle where the 4mm punch biopsies were collected. At day 90, patients with complete re-epithelialization of lesions were considered cured (n=14), and those with active lesions were determined to have failed therapy and subsequently underwent an additional round of antimony treatment (n=7). (B) Volcano plot showing 250 ViTALs according to treatment outcome. Dashed lines indicate $P<0.05$ and $P<0.01$. Each point indicates a gene and point colors reflect the cluster assignment from Fig. 2. Purple text identifies genes found to be differentially over expressed between antimony treatment outcomes in an independent study ($P<0.05$). FC, fold change.

Fig. 4. Elevated expression of cytotoxic genes is associated with higher predicted abundance of CD8+ effector T cells and NK cells in lesions from patients for whom treatment failed. ImmQuant and the DMAP database were used to predict the relative abundance of cell subsets in biopsies based on RNA-seq data. **(A)** Relative cell abundance scores (ImmQuant scores) of CD4+ effector T cells (CD4+CD62L-CD45RA-), CD8+ effector memory T cells (CD8+CD62L-CD45RA-), CD8+ effector T cells (CD8+CD62L-CD45RA+) and NK cells (CD56+CD16+CD3-) between samples (n=21), where each point corresponds to an individual patient sample. Mean and standard deviation are shown. **(B)** ImmQuant scores between the patients who failed the treatment versus patients who cured. Mann-Whitney U tests was used for statistical analysis. **(C)** Correlation matrix between the ImmQuant scores of CD4+, CD8+ effector cells and NK cells between samples and the expression of *GZMB*, *PRF1*, *GNLY*, *CCL3*, *CCL4* and *CCR5*. **(D)** Cells isolated from an independent set of lesions of *L. braziliensis*-infected patients were stained directly ex vivo for flow cytometric analysis prior to treatment with pentavalent antimony. Each point represents a patient and the red ellipse clusters patients (95% confidence) for whom antimony treatment was unsuccessful. Spearman's test was used for correlation statistical analysis and ρ values are represented. ρ , Spearman's rho correlation coefficient; * $P < 0.05$ and ** $P < 0.01$.

Fig. 5. Parasite load correlates with gene expression variability and increased abundance of CD8+ T cells and NK cells.

(A) Relative number of *L. braziliensis* transcripts detected in RNA-seq data from CL lesions (n=21) and healthy skin (n=7, HS). The horizontal line marks the median. (B) Absolute number of parasites detected in 4 mm punch biopsies from lesions and HS, as measured by qPCR. Filled points represent samples with detectable parasite transcripts based on RNA-seq analysis shown in panel A. Spearman's correlation between the absolute number of parasites quantified by in each lesion biopsy by qPCR and (C) abundance of *L. braziliensis* transcripts by RNAseq, or (D) expression of ViTALs, or (E) ImmQuant scores for CD8+ effector T cells and NK cells. Two samples with undetectable parasites were excluded from the correlation plots. ρ , Spearman's rho correlation coefficient; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 6. Parasite load predicts treatment outcome.

(A) Absolute number of parasites per 4mm biopsy and (B) relative abundance of *L. braziliensis* transcripts in patients that were cured versus those for whom treatment was unsuccessful, for the current dataset and the 2016 dataset (28). (C) Kaplan-Meier curve showing time to cure from initial clinical screening. Patients were grouped by those with less (blue) or more (red) than 3.2×10^4 parasites per biopsy, as measured by qPCR.

** $P < 0.01$, Log-rank (Mantel-Cox) test. Mann-Whitney U test, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$