Interplay of human macrophage response and natural resistance of *L.* (*V.*) *panamensis* to pentavalent antimony

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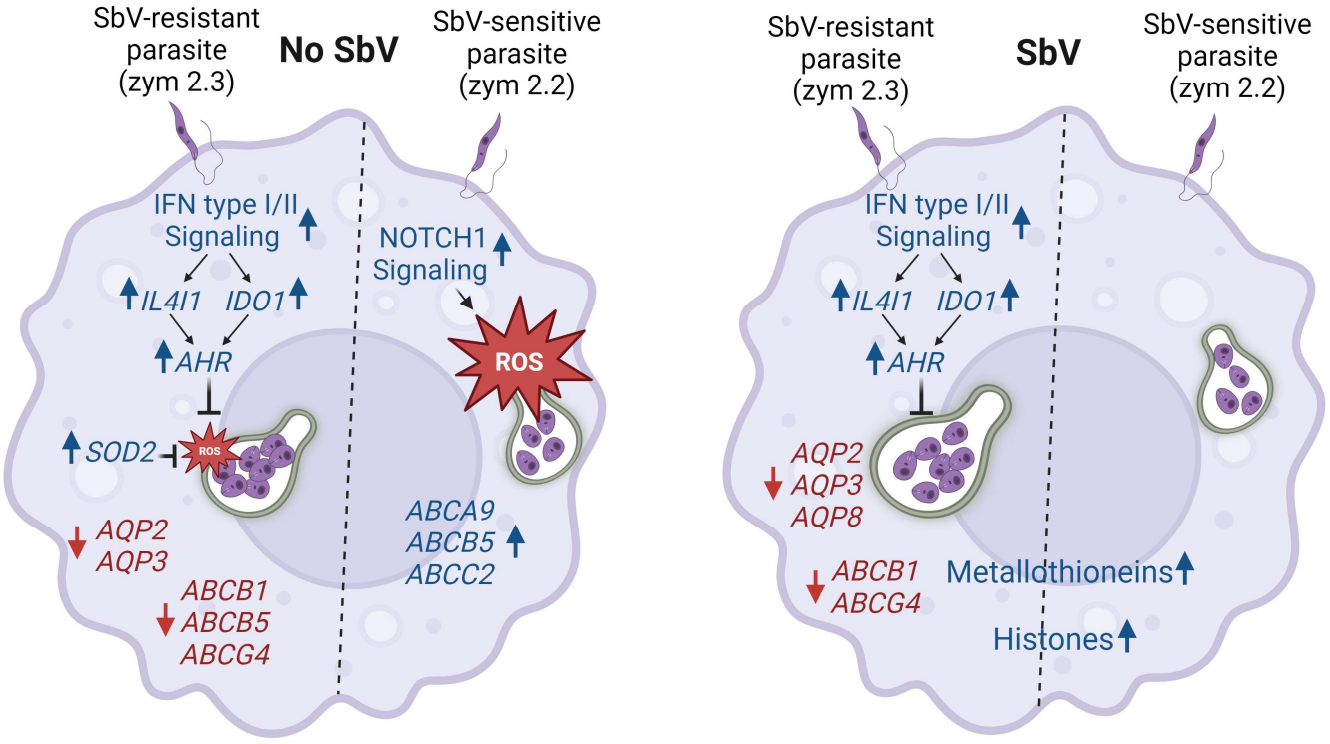
ABSTRACT

Macrophages are the principal host cells of *Leishmania spp.* in human infection and play a critical role in controlling infection and enabling parasite survival and persistence. Nevertheless, understanding of drug resistance in leishmaniasis has primarily focused on the parasite. This investigation provides evidence of the significant differential macrophage response to *ex vivo* infection with clinical strains of *L. (V.) panamensis* naturally resistant (zymodeme 2.3/zym 2.3) or sensitive (zymodeme 2.2/zym 2.2) to antimonial drug, and the distinct effect of this drug on the activation of macrophages. Transcriptome analysis of infected monocyte-derived macrophages from healthy donors revealed significant interferon responses and cytokine signaling by zym 2.3 strains compared to zym 2.2 strains. Furthermore, in the presence of antimony, macrophages infected with zym 2.3 strains, but not with zym 2.2 strains, significantly increased the expression of genes associated with M-CSF-generated macrophages (M-MØ, anti-inflammatory). Notably, macrophages infected with zym 2.3 strains exhibited elevated expression of genes associated with homeostasis and microbicidal regulation, such as the *IDO1/IL4I1-Kyn-AHR* pathways and superoxide dismutase, and downregulation of transporters like *ABC* and *AQP*, compared to macrophages infected with zym 2.2 strains. Remarkably, the majority of these pathways remained upregulated even in the presence of the strong modulatory effect of antimonial drug. Together, these findings demonstrate that the initial and specific parasite-host interaction influences the *ex vivo* macrophage response to antimony. Identification of key pathways in macrophage responses associated with natural resistance to this antileishmanial, enhances understanding of host-response mechanisms in the outcome of *Leishmania* infection and response to treatment.

IMPORTANCE. Drug resistance and treatment failure are increasingly recognized in human leishmaniasis. Investigation of resistance has predominantly focused on parasite-mediated mechanisms. This study examines the role of host macrophages in natural resistance to antimonial drug. Our findings reveal distinct responses by macrophages infected with *Leishmania (Viannia) panamensis* strains that are naturally resistant to antimonial drug versus sensitive strains, both in the presence and absence of the drug. Distinctively, resistant parasites induced regulatory pathways that modulate inflammatory responses and alter host cell transporter expression, potentially contributing to parasite survival under antimony exposure. The host cell-parasite interaction in the context of drug resistant intracellular infections presents opportunities for innovative therapeutic strategies targeting host cell responses.

Graphical abstract

- Clinical strains of *L. (V.) panamensis* naturally resistant or sensitive to antimonial drug (SbV) induce different profiles of human macrophage activation.
- *L. (V.) panamensis* strains with natural SbV resistance induce a significant interferon response in macrophages, accompanied by overexpression of the *IDO1*/*IL4I1-Kyn-AHR* pathways and *SOD2*, associated with immune homeostasis and regulation of microbicidal activity.
- Homeostatic regulation by the *IDO1*/*IL4I1-Kyn-AHR* pathways, induced in macrophages by infection with naturally SbV-resistant strains of *L. (V.) panamensis*, prevails despite significant modulation of macrophage activation by antimony exposure.
- Antimony treatment promotes a more anti-inflammatory (M-M∅) profile in macrophages infected with naturally resistant *L. (V.) panamensis* strains, while macrophages infected with sensitive strains maintain a more proinflammatory profile (GM-M∅).
- Infection with the zym 2.3 strains in the presence of SbV leads to downregulation of specific macrophage transporter genes, supporting the capacity of these naturally SbV-resistant parasites to elicit macrophage responses that enable antimony resistance



INTRODUCTION

Cutaneous leishmaniasis (CL) is a neglected tropical disease that principally affects poor populations around the world. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia (WHO). The pentavalent antimonial drug (SbV), meglumine antimoniate, continues to be the standard of care for CL in most endemic regions worldwide; however, treatment failure is recognized as a significant clinical and public health challenge (1–3). The efficacy of SbV treatment varies by geographic region and the species causing the infection (4–6). The vast majority of cases of CL in Colombia are caused by *Leishmania (Viannia) panamensis* (7–9). Previous investigations by our group documented a persistent natural disparity in susceptibility to antimonial drug between the two most prevalent zymodemes of *L. (V.) panamensis* in Colombia as evaluated by *in vitro* assays (10). The contribution of antimonial drug resistance in treatment failure has been elusive, however, recent studies conducted by our group demonstrated a significantly higher failure rate among patients infected with naturally SbV-resistant *L. (V.) panamensis* zym 2.3 strains, than SbV sensitive zym 2.2 and zym 2.1 strains (11).

Mechanisms of resistance to antimony or other antileishmanial drugs in laboratory-selected strains subjected to sustained exposure to high concentrations of drug, have not been found to reproduce or explain resistance in clinical strains (12,13). The investigation of resistance mechanisms in clinical strains has been limited and with the exception of a few reports (14–16) neither the acquired nor the natural basis of drug resistance detected in clinical strains by *in vitro* evaluation of susceptibility to antileishmanial drugs has been ascertained. Furthermore, the focus of investigation of resistance mechanisms has primarily been on the parasite (16–18) with scant consideration of potentiating mechanisms of parasite survival of drug exposure during parasite-host cell interaction.

Macrophages play crucial roles as host, effector and immunoregulatory cells during *Leishmania* infection (19) and can either eliminate or enable parasite survival, depending on specific and complex interactions between the parasite and the host cell (20,21). Previous investigations have shown that strains of *L. (V.) panamensis* isolated from patients with chronic disease induced significantly higher chemokine gene expression than strains from self-healing cutaneous leishmaniasis (21). Additionally, genes relevant to transport, accumulation and metabolism of antimonials were differentially modulated by antimony sensitive versus resistant strains of *L. (V.) panamensis* (16). In this context, we recently investigated the impact of *L. (V.) panamensis* of zym 2.3 and 2.2 on *ex vivo* human neutrophils. The results showed that the zym 2.3 strain significantly altered the expression of neutrophil genes, increasing the expression of detoxification pathways and reducing the production of cytokines (22). Understanding of the bases of natural drug resistance and the participation of the host cell-parasite interaction, are critical to the identification of more effective host cell targets and for the development of effective therapeutic strategies.

This investigation was conducted to identify host cell responses elicited by infection with naturally resistant zym 2.3 strains of *L. (V.) panamensis* in human macrophages that potentiate or contribute to the diminished susceptibility to antimonial drug associated with treatment failure. To achieve this, transcriptomic profiles of monocyte-derived macrophages following *ex vivo* infection with *L. (V.) panamensis* strains of zym 2.3 and zym 2.2 in the presence or absence of

antimonial drug were compared. Our findings reveal that sensitive and naturally antimony-resistant strains of *L. (V.) panamensis* elicit distinct states of macrophage activation and responses to antimonial exposure. Resistant parasites of zym 2.3 induce regulatory pathways of inflammatory macrophage responses and, in the presence of antimony, promote polarization of macrophages to an anti-inflammatory (M-M∅) profile. The results of this investigation contribute to the identification of targetable host cell responses for innovative treatments that may mitigate the clinical consequences of natural antimony resistance.

RESULTS

Study design

In order to elucidate the participation of host macrophages in the natural resistance to antimony of the genetically and phenotypically distinct zymodeme 2.3 subpopulation of *L. (V.) panamensis*, we investigated the *ex vivo* macrophage response to parasite infection and exposure to pentavalent antimony. Our study utilized distinct and unique subpopulations of clinical strains of *L. (V.) panamensis* exhibiting natural resistance or sensitivity to pentavalent antimony (zym 2.3 and 2.2) (10), as well as monocyte-derived macrophages (MDMs) obtained from healthy donors. A comprehensive analysis of the modulation of pathways in the macrophage response was conducted through transcriptome analysis using RNA-seq **(Fig.1A)**.

A set of six clinical strains of *L. (V.) panamensis*, three naturally sensitive (zym 2.2) and three resistant (zym 2.3) to antimony, were assessed in MDMs obtained from three healthy donors, taking into consideration donor-related variability. To determine parasite-dependent reproducibility of the transcriptomic expression profiles, a different set of six clinical strains (3 each of zym 2.2 and zym 2.3) were evaluated in MDMs of an additional healthy donor. The significant differences observed in the transcriptome profiles across four donors are robust and confirmed that our sample size was sufficient. Macrophage ROS production was evaluated as a potential resistance mechanism based on the identified activation pathways.

Diverse human macrophage activation profiles were induced by antimony exposure and by infection with natural antimony resistant or sensitive strains of *L. (V.) panamensis*

Analysis of global gene expression in human MDMs infected with *L. (V.) panamensis* using principal component analysis (PCA) showed the most pronounced separation (along PC1) between macrophages exposed to antimony versus those that were untreated **(Fig. 1B)**, regardless of infection status. Also evident was the separation (along PC2) of macrophages infected with strains of zym 2.3 from those infected with zym 2.2 strains. Remarkably, the same PCA revealed that the gene expression profiles of macrophages infected with the zym 2.2 of *L. (V.) panamensis* clustered together with those of uninfected cells both in the absence or presence of antimony.

The effect of individual donors was a significant source of variance. When the analysis incorporated a model including donor, zymodeme of infecting parasite, and drug, the majority of variance in the data could not be explained by any of these factors. However, significantly more variance was attributed to donors than to any other factor (**Fig. S1A**). In contrast, when PCA

was performed with donor as the primary factor, the result suggested that drug treatment was the dominant factor in the data, followed by infecting zymodeme (**Fig. S1B**).

Naturally antimony-resistant strains of *L. (V.) panamensis* elicit a pronounced inflammatory response in conjunction with regulatory pathways

Differential expression (DE) analyses comparing infected and uninfected macrophages revealed that infection by zym 2.3 strains induced a more profound perturbation of the MDM transcriptome than zym 2.2 strains, modulating 646 genes vs. 268 genes, respectively ($log_2FC \geq 1$ or ≤ -1; $P_{adj} < 0.05$) (**Fig. 2D**). Remarkably, the transcriptomic responses of MDMs to infection with zym 2.3 vs. zym 2.2 strains were distinct and quite unique, sharing only 88 up- or downregulated genes. Nine genes exhibited opposing expression patterns in macrophages infected with zym 2.3 and zym 2.2 strains (*ABCB5, RFX4, CA14, EGR1, MCF2L, DNASE1L3, FOS, IFITM10, and PKD1L3*) being downregulated in zym 2.3-infected macrophages and upregulated in zym 2.2-infected macrophages. This set of genes encompasses a wide range of cellular functions, including ATP-dependent transmembrane transport, transcriptional regulation, cell proliferation and differentiation. Some of the genes are associated with skin diseases and antimony transport (*ABCB5*) (23), the innate immune response to *Leishmania* (*FOS*) (24), and parasite survival (*EGR1*) (25).

For each comparison carried out, we have illustrated the differential gene expression profiles with volcano plots (**Fig. 2A-C**), labeling a selection from the significant top 10 up- or downregulated genes. An examination of the lists of differentially expressed genes revealed genes associated with several biological processes, highlighting upregulated genes associated with inflammatory response (*PTGES, CCL8 and EREG*), in the comparison between macrophages infected with zym 2.3 strains and uninfected cells (**Fig. 2A**), as well as in the comparison between macrophages infected zym 2.3 strains and those infected with zym 2.2 strains (**Fig. 2C**). Most remarkably, the expression of *IDO1* (indoleamine 2,3-dioxygenase 1) ranked among the top ten genes that were significantly induced by infection with zym 2.3 strains (**Fig. 2A and C**). IDO is a key enzyme in tryptophan catabolism that depletes this amino acid and generates kynurenines, creating an immunosuppressive microenvironment and dampening macrophage proinflammatory responses.

Pathway enrichment analyses based on a set of significantly upregulated genes ($log_2FC \geq 1$; $P_{adj} < 0.05$) in macrophages, showed that *L. (V.) panamensis* strains of zym 2.3 induce more activation and regulation of immune response compared to both uninfected and zym 2.2-infected macrophages (**Table S3**, **Fig. 2E**). This inflammatory immune response is evidenced particularly by enhanced cytokine signaling (*CCL8*, *CCL4*, *CCL5*, *CCL1*, *CXCL8*, *CXCL3* and *CXCL5*) and interferon responses. Key pathways include interferon alpha/beta and gamma signaling, antiviral mechanisms mediated by IFN-stimulated genes, and chemokine receptor interactions. Interestingly, pathways associated with the negative regulation of inflammatory macrophage responses are also upregulated by infection with zym 2.3, including the tryptophan catabolic process, and negative regulators of DDX58/IFIH1 signaling, two key components of the innate immune response to viruses (**Table S3)**. Key genes associated with the regulation of oxidative stress (i.e. *SOD2*) and the regulation of the inflammatory response (*AHR, IDO and IL4I1*) (26,27) were also overexpressed in macrophages infected with zym 2.3 strains (**Fig. 2E**) (Graphical abstract). On the other hand, macrophages infected with zym 2.2 strains, compared with

uninfected cells, induce a unique set of pathways that distinctly differed from those observed in zym 2.3 infection. These pathways are primarily related to developmental signaling and cellular differentiation, with a particular influence on NOTCH1 signaling (**Table S3, Fig 2E**). This repertoire of pathways presents a striking comparison to the immune response of macrophages observed during zym 2.3 infection.

The pathway enrichment analysis based on downregulated genes revealed distinct patterns for zym 2.3 and zym 2.2 infections. In contrasts between macrophages infected with zym 2.3 and uninfected cells, downregulated genes (n = 253) were associated with ABC transporter activity, endocytosis, phagocytosis, and intracellular trafficking. Notably, only six genes were downregulated in the comparison between zym 2.2 infection and uninfected cells. When comparing zym 2.3 to zym 2.2 infections, macrophages infected with zym 2.3 were characterized by downregulation of genes associated with ABC (*ABCB1*, *ABCB5* and *ABCG4*) and AQP (*AQP2* and *AQP3*) transporters, NOTCH1 signaling, pathways involved in specific immune responses (particularly interleukin-8 and interleukin-2 related processes), and cellular differentiation and growth. These findings suggest that zym 2.3 infection has a more profound effect on suppressing various host cell processes compared to infection by zym 2.2 strains.

Infection with natural antimony-resistant strains of *L. (V.) panamensis* sustains regulatory mechanisms of the macrophage inflammatory response despite significant modulation caused by antimonial drug

The differential gene expression profiles of macrophages infected with *L. (V.) panamensis* of either zymodeme and subjected to antimony treatment, revealed a predominant drug effect that was independent of the parasite zymodeme (**Fig. 1B**). This drug effect was also manifested by the high number of modulated genes (516 downregulated, 306 upregulated) that were shared by uninfected macrophages and those infected with either zym 2.2 or zym 2.3 strains (**Fig. 3E**). In macrophages infected with the zym 2.2 strains, treatment with antimony resulted in the downregulation of a higher number of genes (n=260), compared to the number of genes downregulated during zym 2.3 infection (n=173). Conversely, exposure to antimony resulted in the upregulation of a greater number of genes (n=148) in macrophages infected with zym 2.3 strains compared to those upregulated in macrophages infected with zym 2.2 strains (n=70). The effect of antimony on uninfected macrophages demonstrated significant modulation of both up (n=95) and down (n=131) regulated genes (**Fig. 3E**).

Volcano plots document similarities in the top ten upregulated or downregulated genes in macrophages infected with zym 2.2 and zym 2.3 strains exposed to antimony (**Fig. 3A** and **3B**), as well as in uninfected cells exposed to antimony (**Fig. 3D**). A Spearman rank correlation analysis of the top 50 upregulated and top 50 downregulated genes (100 genes in total) of macrophages infected with zym 2.3 and zym 2.2 in the presence of antimony demonstrated a significant positive correlation ($r = 0.7922$, $P = 0.0001$) substantiating the predominant effect of the drug in the modulation of macrophage response. However, the comparison between macrophages infected with zym 2.3 and zym 2.2 strains in the presence of antimony indicates the conservation of genes exclusively induced by infection with 2.3. These include several interferon-related genes such as *IFI44L, RSAD2, ISG20, OASL, CCL8 and ZBP1*, associated with macrophage activation, as well as *NGFR, OTOF, SLC38A5,* and the immunoregulatory gene *IDO1* (**Fig. 3C**).

Reactome pathway database and Gene Ontology gene enrichment analysis of upregulated genes in macrophages exposed to antimony revealed that more than 80% of the top ten pathways identified are shared among uninfected and infected cells in the presence of the drug, independently of the zymodeme strain (**Table S4**). Some of these pathways are recognized for their potential relevance in antimony metabolism, such as metallothionein binding to metals, response to metal ions, and detoxification of copper ions (28,29). Conversely, a similar analysis of the principal pathways enriched in downregulated genes in the presence of antimony revealed few shared pathways between macrophages infected with zym 2.3 and zym 2.2 strains. These shared pathways are principally related to connective tissue disorders and eosinophil cell migration. Notably, these pathways were not downregulated in uninfected macrophages exposed to the drug, suggesting that their downregulation is specific to the combined effect of *Leishmania* infection and antimony exposure. The analysis of specific pathways for infection with each of the two zymodemes, compared to uninfected cells and based on the downregulated genes (Table S4), suggest different adaptive mechanisms of parasite regulation of macrophage response. For example, in presence of antimony infection with zym 2.2, but not with zym 2.3, downregulates genes associated with inflammasomes and pro-inflammatory cell recruitment, pointing to a suppression of the acute inflammatory response.

Analysis of pathways based on the specific comparison of macrophages infected with zym 2.3 vs. zym 2.2 in the presence of antimony showed that infection with zym 2.3 induced a marked upregulation of genes associated with an interferon response, including interferon alpha/beta and gamma, similar to the response that was elicited by infection in the absence of the antimonial drug. This included the significantly higher expression of regulatory genes of the inflammatory response such as *IDO1/2*, *IL4I1*, *KYNU*, and *AHR.* Additionally, pathways involved in telomere packaging and DNA repair processes, such as depurination and purine damage recognition, were also upregulated. Enriched Gene Ontology biological processes further highlighted pathways chronic inflammatory responses, and chemokine interactions (**Table S4**). Remarkably, the analyses of genes downregulated by infection with zym 2.3 compared to zym 2.2 in the presence of antimony revealed downregulation of pathways associated with aquaporin-mediated transport (*AQP2, AQP3, and AQP8*) and ABC transporter transcription activity (*ABCB1 and ABCG4*). These findings are particularly significant as aquaporins and ABC transporters have been previously recognized for their role in antimony transport and drug susceptibility (16,29).

Treatment with antimony significantly increases the expression of genes associated with M-CSF-generated Macrophages (M-MO, anti-inflammatory) during infection with naturally antimony-resistant strains.

Based on the gene sets that define the transcriptome of human monocyte–derived proinflammatory GM-MØ (“Proinflammatory gene set”) or anti-inflammatory M-MØ (“Anti-inflammatory gene set”) previously reported (GSE68061) (30), we analyzed and identified a notable over-representation of genes associated with the proinflammatory profile of human macrophages (GM-MØ-specific gene set) across infections with both zymodemes **(Fig. 4A)**. However, a distinct negative enrichment of genes associated with an anti-inflammatory profile (M-MØ-specific gene set) was observed only in infections with the zym 2.3 strains, suggesting a stronger proinflammatory response to these infections compared with zym 2.2 strains. Interestingly, GSEA revealed that antimony modulates the macrophage expression profile in a

manner dependent on the infecting zymodeme. In macrophages infected with zym 2.3 strains, treatment with antimony resulted in a negative enrichment of genes associated with a proinflammatory profile and a positive enrichment of genes associated with an anti-inflammatory profile ((M-MØ-specific gene set; GSE68061) **(Fig. 4B)**. In macrophages infected with zym 2.2, antimony exposure decreased the expression of genes linked to the proinflammatory response without significant changes in genes related to the anti-inflammatory response. Moreover, an evaluation of the effect of antimony on uninfected macrophages indicated a negative enrichment in proinflammatory gene sets, without changes in the expression of anti-inflammatory genes (**Fig. 4C**). These findings underscore the nuanced role of antimony in modulating immunoinflammatory responses, to the strain of *Leishmania* involved.

The positive association of macrophages infected with zym 2.3 strains and the enrichment of genes associated with an anti-inflammatory profile (M-CSF), combined with higher expression of genes associated with regulatory response of inflammatory response and microbicidal activity (*IDO1/2*, *IL4I1*, *KYNU*, and *AHR*), are consistent with the significant parasite burden in infections with zym 2.3 compared to zym 2.2 in the presence of antimony. This was supported by the measurement of gene expression corresponding to intracellular amastigotes (**Fig. S2**).

Reduced production of ROS corresponds with regulatory pathways induced in macrophages infected with naturally SbV-resistant strains of *L. (V.) panamensis*

Oxidative stress is characterized by high levels of reactive oxygen species (ROS), which have direct antimicrobial activity against pathogens and have been reported to be relevant in the microbicidal activity against *Leishmania* infection, depending on the parasite species (31). Interestingly, the results obtained in the present study show that macrophages infected with zym 2.3 exhibit a higher expression of genes and pathways associated with the regulation of oxidative stress and proinflammatory response, such as *SOD2*, the *IDO1*/*IL4I1-Kyn-AHR* pathway, and the *IL4I1*. Because SOD isoforms constitute the major antioxidant defense systems against ROS, (32) and the tryptophan-degrading reaction catalyzed by IDO is linked to the scavenging of ROS, (33) we evaluated the production of these gene products in MDMs from healthy donors. Results demonstrated that human macrophages infected with zym 2.3 of *L. (V.) panamensis* generate significantly lower ROS response compared to macrophages infected with zym 2.2 strains **(Fig. 5)**. Hence, the regulation of ROS production may be a mechanism that favors the survival of the Sb-resistant zym 2.3 of *L. (V.) panamensis*. It should be noted that the induction of ROS is a reported mechanism of antileishmanial activity for antimony (34).

DISCUSSION

This investigation provides a comprehensive transcriptomic analysis of distinct human macrophage responses to *ex vivo* infection with *Leishmania (Viannia) panamensis* strains naturally sensitive or resistant to antimony. Our findings reveal different macrophage activation states induced by infection with sensitive (zym 2.2) and resistant (zym 2.3) strains, both in the absence and presence of antimony. Through the elucidation of macrophage responses in the context of natural antimony resistance, we have identified two principal differences in

343 macrophage activation potentially associated with this resistance: regulation of the inflammatory
344 and microbicidal response, and changes in the expression of host cell transporters. These
345 differences could explain the higher parasite survival of zym 2.3 strains compared to zym 2.2
346 strains in the presence of antimony. Our results provide new insights into the molecular
347 mechanisms underlying antimony resistance in cutaneous leishmaniasis and highlight the
348 complex adaptive interplay between the parasite, host cell, and drug treatment.

349 Analysis of macrophage responses revealed that antimony-resistant zym 2.3 strains
350 induce a more profound perturbation of gene expression compared to sensitive zym 2.2 strains,
351 modulating more than double the number of genes. Notably, zym 2.3 strains activated pathways
352 in macrophages associated with a stronger inflammatory response than zym 2.2, evidenced by
353 upregulation of interferon signaling, inflammatory cytokines, and chemokines. However, this
354 activation profile was accompanied by induction of immune homeostasis and regulatory
355 pathways that modulate the innate microbicidal response, a phenomenon specific infection by
356 zym 2.3 strains. A key finding was the significant upregulation of *IDO1* in macrophages infected
357 with zym 2.3. This gene was among the top ten upregulated genes, with a fold change of 7.1
358 compared to uninfected cells, and 5.9 compared to macrophages infected with zym 2.2 strains.
359 Interestingly, despite the strong effect of antimony on macrophage gene expression, *IDO1*
360 upregulation persisted in zym 2.3-infected macrophages exposed to antimony, maintaining a
361 fold change of 6.1 compared to zym 2.2-infected macrophages in the presence of antimony.

362 IDO is an interferon (IFN-I/II) inducible enzyme whose activity can inhibit the growth of
363 susceptible intracellular pathogens by catalyzing the oxidative cleavage of the indole ring of L-
364 tryptophan and depleting pools of this essential amino acid. However, it can also mediate
365 macrophage homeostasis of proinflammatory response and contribute to pathogen survival
366 through induction of metabolites, production of regulatory cytokines, and control of oxidative
367 stress (35). Enrichment analysis revealed overexpression of other IDO pathway-associated
368 genes in zym 2.3-infected macrophages, including *IDO1/2*, and *AHR*. Notably, the *IDO1-Kyn-*
369 *AHR* pathway has been described as a survival mechanism for other intracellular pathogens,
370 including *Mycobacterium tuberculosis* and *Toxoplasma* (36). Previous studies have shown that
371 upregulation of IDO by *L. donovani* and *L. major* can favor parasite persistence in BALB/c mouse
372 macrophages and local lymph nodes, and IDO inhibition reduced both local inflammation and
373 parasite burden (37,38). In contrast, studies in *L. (V.) panamensis*-infected BALB/c mice showed
374 that blocking IDO exacerbated the disease, enlarging lesions and increasing the parasite load
375 (39). However, in lesions from patients with cutaneous leishmaniasis infected with *L. (V.)*
376 *panamensis* or *L. (V.) braziliensis*, *IDO1* expression was downregulated during healing in chronic
377 patients and positively correlated with IFNγ expression (40). These latter findings align with our
378 results in macrophages, where those infected with zym 2.3 strains overexpressed genes related
379 to interferon response and high expression of *IDO1*.

380 Interleukin 4-induced gene 1 (*IL4I1*) was also significantly upregulated in macrophages
381 infected with zym 2.3, both in the absence and presence of antimony. IL4I1 belongs to the L-
382 amino-acid oxidase (LAAO) family and catalyzes the oxidation of tryptophan. It contributes to
383 the suppressive activities of macrophages and promotes differentiation of alternatively activated
384 M2 macrophages with anti-inflammatory properties (41). Similar to *IDO1*, *IL4I1* expression can
385 create a local anti-inflammatory microenvironment. High levels of IDO1 and IL4I1 mediate aryl
386 hydrocarbon receptor (AHR) activation through tryptophan catabolism. AHR is a ligand-activated
387 transcription factor that regulates various biological processes, including immune modulation.

Both IDO and IL4I1 have been associated with immunosuppression in tumors through the generation of the AHR agonist kynurenic acid (KynA), further underscoring the importance of these molecules in AHR signaling. These two enzymes have been proposed as concomitant targets to achieve derepression of anti-tumor immunity (27). The co-expression of *IDO1* and *IL4I1* as activators of AHR supports the potential participation of the *IDO1*/*IL4I1-KYN-AHR* pathways in the antimicrobial of macrophages infected with zym 2.3 strains.

In concurrence with the overexpression of genes of the *IDO1*/*IL4I1-KYN-AHR* pathways and its association with anti-microbicidal responses, macrophages infected with zym 2.3 significantly overexpressed superoxide dismutase 2 (*SOD2*) gene compared to both uninfected macrophages and those infected with zym 2.2 strains. SOD isoforms are the major antioxidant defense systems against reactive oxygen species (ROS) (32), one of the principal antimicrobial mechanisms against pathogens (42). Our functional studies on ROS production corroborated the transcriptomic findings. Macrophages infected with zym 2.3 strains generated significantly lower ROS responses compared to those infected with zym 2.2 strains. Similar results were observed in neutrophils exposed *ex vivo* to infection with zym 2.3 and zym 2.2 strains, where zym 2.3 strains induced significantly lower ROS production compared to zym 2.2 strains, both in the presence and absence of SbV (43). This reduced ROS production in zym 2.3-infected macrophages may represent one of the mechanisms favoring parasite survival, and antimony resistance of zym 2.3. Overexpression of *SOD2*, in conjunction with other mechanisms regulating macrophage activation, such as the activation of the *IDO1*/*IL4I1-KYN-AHR* pathways, could contribute to natural antimony resistance in zym 2.3 infection. However, our results showed that the addition of antimonial drug significantly reduced *SOD2* expression in macrophages infected with zym 2.3 compared to infection in the absence of antimonial drug. Functional assays of these pathways are needed to define their precise role in the diminished response of the naturally resistant zym 2.3 subpopulation to antimonial drug.

The differences in the activation profiles of macrophages elicited by infection with zym 2.2 and zym 2.3 of *L. (V.) panamensis* not only define the macrophage response to infection but also have implications for the *ex vivo* and significantly associated *in vivo* therapeutic activity of antimony. Remarkably, the addition of antimony to macrophages infected with zym 2.3 significantly increased the expression of genes associated with M-CSF-generated macrophages (M-MØ, anti-inflammatory), as revealed by Gene Set Enrichment Analysis based on ranked comparison of the transcriptomes of M-MØ versus GM-MØ (GSE68061). This shift towards an anti-inflammatory phenotype (M-MØ) in the presence of antimony could potentially contribute to drug tolerance/resistance by creating a more permissive environment for parasite survival. Similar results have been observed with other intracellular pathogens, such as *Mycobacterium tuberculosis* and *Salmonella typhimurium*. *Mycobacterium tuberculosis* infection can induce both M1 and M2 macrophage polarization, but M2 macrophages are more abundant in chronic infections (44,45). Treatment of *Mycobacterium tuberculosis*-infected macrophages has been shown to promote M2 polarization, which may contribute to the lower susceptibility to antimicrobial drug (46). *Salmonella typhimurium* infection can induce both M1 and M2 macrophage polarization (47), but M2 macrophages are more abundant in drug resistance. Overall, these results suggest that the differential modulation of macrophage polarization by *Leishmania* infection may be a common feature of intracellular pathogens and this adaptive capacity has important implications for the development of novel, more effective immunomodulatory and antimicrobial therapies.

In contrast, transcriptome analysis of macrophages infected with zym 2.2 strains highlighted the role of NOTCH signaling 1 in the macrophage response. NOTCH-1 signaling is known to promote M1 polarization through reprogramming of macrophage metabolism, characterized by high production of mitochondrial ROS (48). This aligns with pro-inflammatory profile (GM-CSF) of macrophages generated during infection with zym 2.2, as identified by GSE analysis, and concurs with the higher ROS production observed in these macrophages compared to those infected with zym 2.3 strains. On the other hand, addition of antimony significantly downregulated the genes associated with NOTCH 1 signaling, resulting also in a negative enrichment of gene expression associated with a pro-inflammatory profile (GM-CSF). In contrast to the infection with zym 2.3 strains, the addition of antimony macrophages infected with zym 2.2 did not increase the expression of genes associated with an anti-inflammatory profile (M-CSF). These findings highlight the distinct activation profiles of macrophages infected by zym 2.2 and 2.3 strains, both in the presence and absence of antimony.

Despite the differences induced by antimony exposure in the polarization profile of macrophages infected with zym 2.2 and 2.3 strains, we found that that 80% of the top ten pathways identified were shared among uninfected and infected cells and therefore, associated with the effect of antimony. These pathways are principally related to cellular mechanisms to counteract and prevent metal-induced toxicity, as evidenced by the enrichment of pathways involving metallothionein binding to metals, response to metal ions, and detoxification of copper ions. The participation of metallothioneins in the response to antimony in macrophages infected with *Leishmania* has been previously reported and associated with facilitating Sb-dependent killing of *Leishmania* (28). These results suggested that metallothioneins scavenging function could promote Sb accumulation within the phagolysosome of infected macrophages, favoring intracellular killing of *L. (V.) panamensis* (29). Additionally, it is recognized that metallothioneins play a crucial role in cellular redox balance, supporting their participation in the reduction of the pentavalent antimony pro-drug to the trivalent form having greater antileishmanial activity. These results are consistent with the significant reduction of parasite burden by exposure to antimony during *ex vivo* infection both zym 2.3 and 2.2 strains. However, independent of the marked effect of antimony on the transcriptome of macrophages, according to the GSE analysis classification, the anti-inflammatory profile of macrophages infected with zym 2.3, could explain the significant intracellular parasite survival of zym 2.3 strains in the presence of antimonial drug.

Interestingly, our analysis revealed distinct patterns in the expression of transporter genes by macrophages during zym 2.3 and zym 2.2 infections. Notably, infection with zym 2.3 in comparison with zym 2.2, in the presence of antimony led to the downregulation of pathways associated with aquaporin-mediated transport (*AQP2*, *AQP3*, and *AQP8*) and ABC transporter activity (*ABCB1* and *ABCG4*). Given the known role of these transporters in antimony uptake and drug susceptibility, these findings provide insight into potential mechanisms of antimony resistance in zym 2.3 strains. The downregulation of aquaporins could potentially reduce antimony influx into the cell, while the reduced expression of ABC transporters might alter the intracellular distribution of the drug. These changes in transporter expression, combined with the anti-inflammatory profile (M-CSF) induced by zym 2.3 in the presence of antimony, could create a cellular environment that enables parasite survival despite exposure to leishmanicidal concentrations of antimonial drug. Furthermore, the differential regulation of these transporters in zym 2.3 and zym 2.2 infections underscores the subpopulation-specific adaptations that may contribute to antimony resistance and treatment failure.

In conclusion, our study reveals that naturally SbV-resistant *L. (V.) panamensis* zym 2.3 strains elicit a unique macrophage response characterized by a strong but regulated inflammatory profile, reduced ROS production, and altered expression of drug transporters. These factors likely contribute to the generation of an intracellular environment favoring parasite survival thereby participating in resistance to the antileishmanial effect of antimonial drug. Understanding the interplay of complex host-parasite interactions provides valuable insights into the bases, and mechanisms of resistance to this widely used anti-leishmanial and guides the development of new therapeutic strategies for leishmaniasis and other intracellular pathogens.

MATERIALS AND METHODS

Study population and ethics statement. Transcriptome analysis and ROS production were performed using MDMs obtained from nine healthy volunteers, both male and female, between 18 and 60 of age, with no clinical history of leishmaniasis. Volunteers resided in the municipality of Cali, Colombia, Recruitment was conducted by the clinical support team of the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Cali. A blood sample of 200 mL was collected from each volunteer. The study was approved and monitored by the CIDEIM Institutional Review Board for research involving human subjects in accordance with national and international guidelines for Good Clinical Practice. Every participant provided voluntary, informed, and signed consent.

Clinical Strains. Twelve *Leishmania (Viannia) panamensis* strains isolated at the time of diagnosis from patients with cutaneous lesions by medical personnel in CIDEIM, and cryopreserved in liquid nitrogen in the institutional biobank were included in this study. Six strains belonging to the zym 2.2 and six strains of zym 2.3 that had been evaluated for antimonial drug susceptibility, and species confirmed serodeme and zymodeme (7,49) were used for *ex vivo* assays within four passages from isolation. Species identification was achieved by isoenzyme electrophoresis (49) and indirect immunofluorescence using species-specific monoclonal antibodies as described elsewhere (7). Drug susceptibility of intracellular amastigotes was estimated by evaluation of percentage of parasite survival in PMA-differentiated U-937 cells (ATCC CRL-1593.2) after exposure to pentavalent antimony (SbV) at a final concentration of 32 µg/mL, compared to control without drug exposure (50).

Culture of monocyte–derived macrophages, infection, and drug exposure. Monocytes were isolated from peripheral blood samples (200 mL) obtained from healthy donors by centrifugation over a Ficoll-Hypaque gradient (Sigma-Aldrich, 10771; USA) followed by purification with magnetic anti-CD14 microbeads (Miltenyi Biotec, LS Columns 130-042-401; USA), according to the manufacturer's instructions. Macrophage cell cultures were generated by adherence of monocytes (>95% CD14+ cells) to cell culture plasticware in a 6-well plates (1 x 10^6 cells in 3 ml of medium per well) in serum-free RPMI 1640 medium (Gibco, 22400; USA) for 2 h, followed by culture for 7 days in RPMI 1640 media supplemented with 20% heat inactivated FBS (Gibco, 10082; USA) at 37°C in 5% CO_2 [16]. Infection with *Leishmania* promastigotes was performed at a ratio of 5 parasites per macrophage. Prior to infection, parasites were opsonized with 10% AB positive human serum (Sigma-Aldrich H3667; USA).

Infected cells were incubated for 24 hours at 37°C in 5% CO_2, and then exposed to additive-free meglumine antimoniate (Walter Reed 214975AK; lot no. BLO918690-278-1A1W601; antimony analysis, 25% to 26.5%, by weight) at a final concentration of 32 µg SbV/ml for 72 hours as previously described (50). The concentration of 32 µg SbV/ml, corresponds to the estimated maximum plasma concentration (plasma C_{max}) during standard-of-care-treatment (51). Infected and uninfected macrophages with and without antimony treatment were included for all assays.

Transcriptomic profiling and data analysis of differentially expressed genes. Macrophages cultured under the described experimental conditions were collected using Trizol, treated with DNAseI, and purified using the RNeasy mini kit (Qiagen, 74704; USA). RNA integrity was assessed using an Agilent 2100 bioanalyzer (RIN ≥ 9). Poly(A)-enriched cDNA libraries were generated using the Illumina TruSeq sample preparation kits and quality and quantity verified using the bioanalyzer and qPCR (KAPA Biosystems). Using dual-index barcoding methods to reduce index hopping, we multiplexed up to 20 samples per NextSeq 1000 P2 run to ensure sufficient read depth (20M 60 bp paired-end reads) per sample. Sequence quality metrics were assessed using FastQC and Trimmomatic (52) was used to remove adapter sequences and trim when the mean quality score fell below 25. Reads were aligned against the human genome as well as the *L. (V.) panamensis* (TriTrypDb release 36) using hisat2 (53). The resulting alignments were sorted and indexed via samtools (54) and passed to htseq (55) for generating count tables. Genes with <2 reads per million in n samples where n is the size of the smallest group of replicates (56) were removed excluded from the analysis prior to abundance estimation and subsequent differential expression analyses. All sequences generated were submitted to the Short Read Archive (SRA) at the NCBI (BioProject: PRJNA1156057).

Reactive Oxygen Species (ROS) Production. ROS production was measured using a luminol-based chemiluminescence assay, as previously described (43). Monocytes from five healthy donors were resuspended in an RPMI medium at 5 x 10^5 c/ml and distributed into the wells (100 µL) of white opaque 96-well microplates (Perkin Elmer, PKE_6005299). Macrophage cell cultures were generated by adherence of monocytes to cell culture plasticware as previously described (16). Cells were cultured in HBSS (Sigma-Aldrich, H668; USA) infected with clinical strains of *L. (V.) panamensis* at a ratio of 5 parasites per macrophage, and ROS production was evaluated immediately for one hour. Luminol Sodium Salt (Carbosynth Limited) was added prior to infection at a final concentration of 20 µg/ml, and luminescence induced by ROS was measured every 2.5 min over 60 min using a plate reader (Chamaleon). The positive control was the induction of ROS by 100 ng/ml PMA.

Statistical Analysis. Biological replicates and batch effects were assessed and visualized using the hpgltools (https://github.com/elsayedlab/hpgltools) R package. The process included creating density plots, boxplots of depth, coefficient of variance, hierarchical clustering analyses, variance partition analyses (57), and PCA before and after normalization. Combinations of normalization and batch adjustment strategies were evaluated. The normalization methods that were typically tested included trimmed median of M-values, relative log expression, and quantile. These were combined with batch evaluation strategies from the surrogate variable analysis package (sva) (58). Differential expression (DE) analyses were performed using a single pipeline which conducted all pairwise comparisons using the Bioconductor packages: limma, edgeR (59), DESeq2 (60), and EBSeq (61). In each case (except EBSeq), the surrogate variable estimates

provided by sva were used to adjust the statistical model in an attempt to address the batch/surrogate effects. The quality of each comparison was evaluated by the degree of agreement among methods, but the interpretations were likely primarily informed by the DESeq2 results. Pairwise contrasts in genes with a Benjamini-Hochberg multiple-testing adjusted *P* value of < 0.05 were defined as differentially expressed. Genes with significant changes in abundance (false discovery rate adjusted P values ≤ 0.05) were passed to gProfileR2 (62), KEGG pathway analyses using ConsensusPathDB (63), and gene set variation analysis (GSVA) (64). Gene ontology analyses were supplemented with manual data curation. The Mann–Whitney U test was used to establish statistical differences between ROS production induced in macrophages by infection with 2.2–sensitive and 2.3-resistant strains. The code used to perform the various analyses described in the manuscript is fully accessible on GitHub at https://github.com/elsayed-lab/macrophage_response_lpanamensis.

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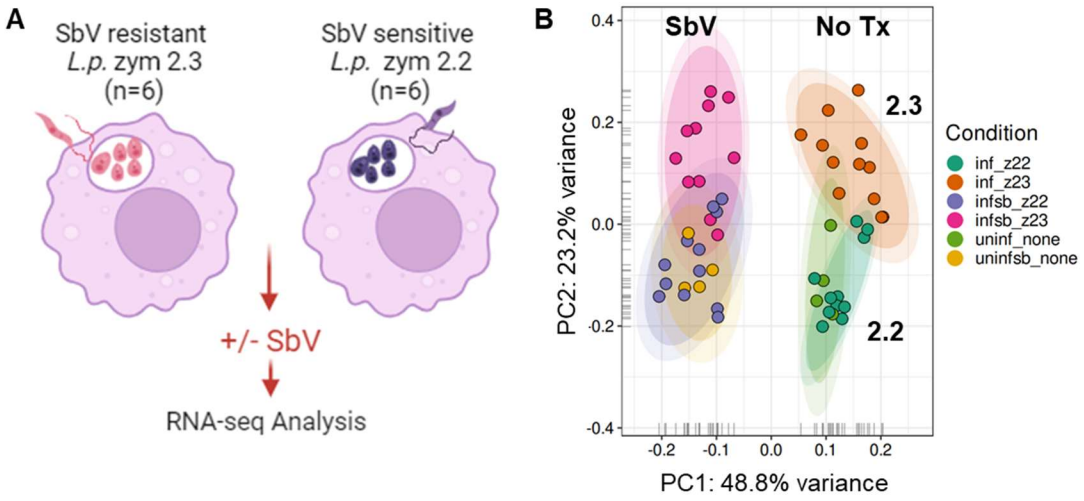

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800 **Figure 1. Global transcriptome profiling of human MDMs infected with zym 2.2 and zym 2.3 of *L. (V.)***
801 ***panamensis* in absence or presence of antimony. A.** Schematic of experimental strategy **B.** Principal component
802 analysis (PCA) plot of normalized and SVA-adjusted RNA-seq expression values of macrophage samples from 4
803 donors, uninfected (none) or infected with strains of zym 2.2 and zym 2.3, in the absence or presence of 32 µg
804 SbV/mL.
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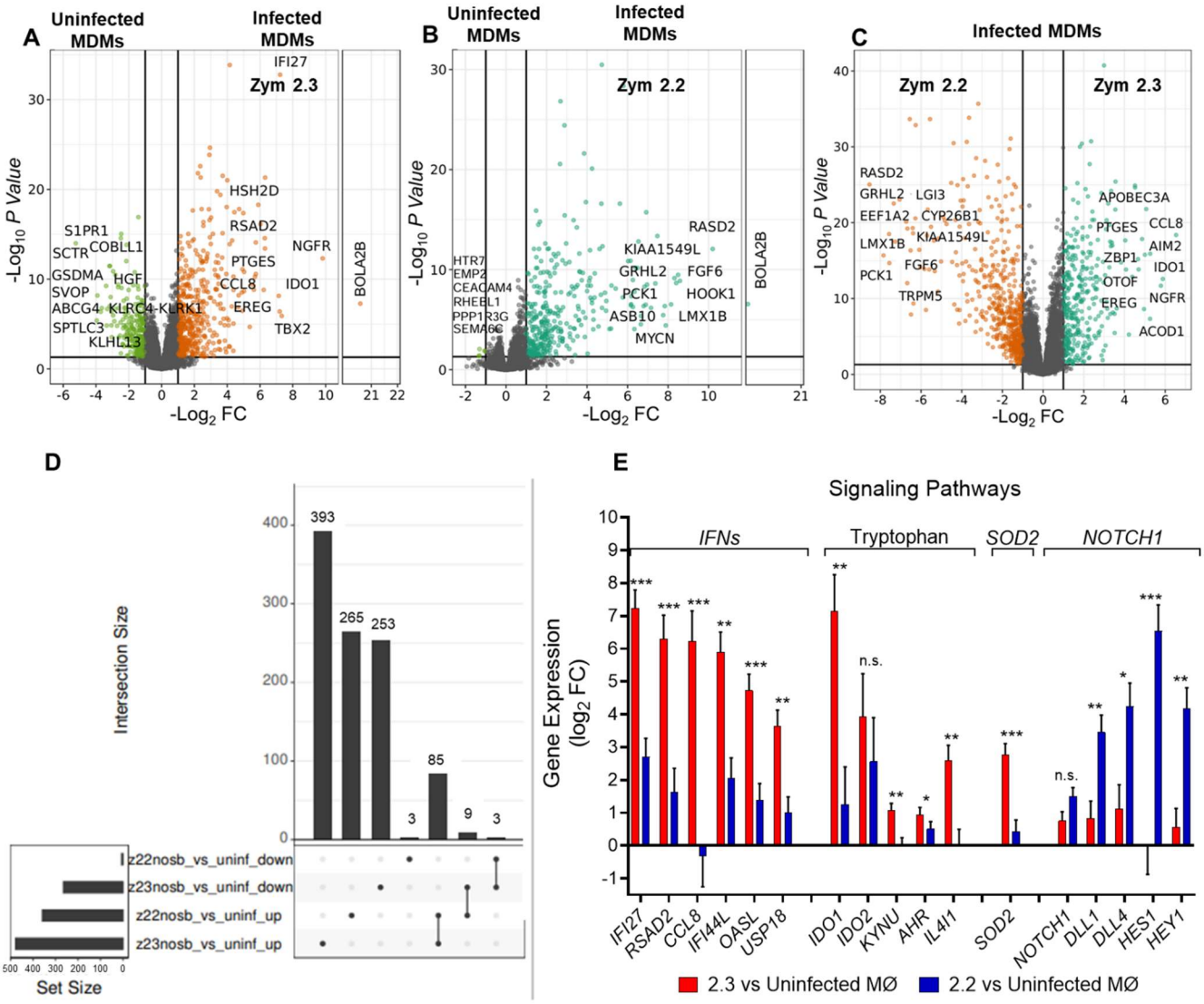

Figure 2. Differentially expressed genes in macrophages infected with *L. (V.) panamensis* zym 2.2 or zym 2.3. Volcano plots visualizing comparisons of **A.** MDMs infected with zym 2.3 strains vs. uninfected cells; **B.** MDMs infected with zym 2.2 strains vs. uninfected cells; **C.** MDMs infected with zym 2.3 strains vs. MDMs infected with zym 2.2 strains. The top 10 up or downregulated genes are labeled in panels **A-C** when space allows, and the color scheme is analogous to the one used in Fig.1. **D.** UpSet plot summarizing the numbers of unique and shared upregulated and downregulated genes by infection with zym 2.3 and zym 2.2 strains. **E**. Differential expression of highlighted genes from selected pathways in macrophages infected with zym 2.3 strains and zym 2.2 strains of *L. (V.) panamensis.* Data correspond to transcriptome analysis of monocyte-derived macrophages from four healthy donors. Panels A to D: Genes were deemed up- or down-regulated when $\log_2$ FC > 1 or < -1, and adj. $P < 0.05$. * $P < 0.05$ ** $P \leq 0.01$, *** $P \leq 0.001$.

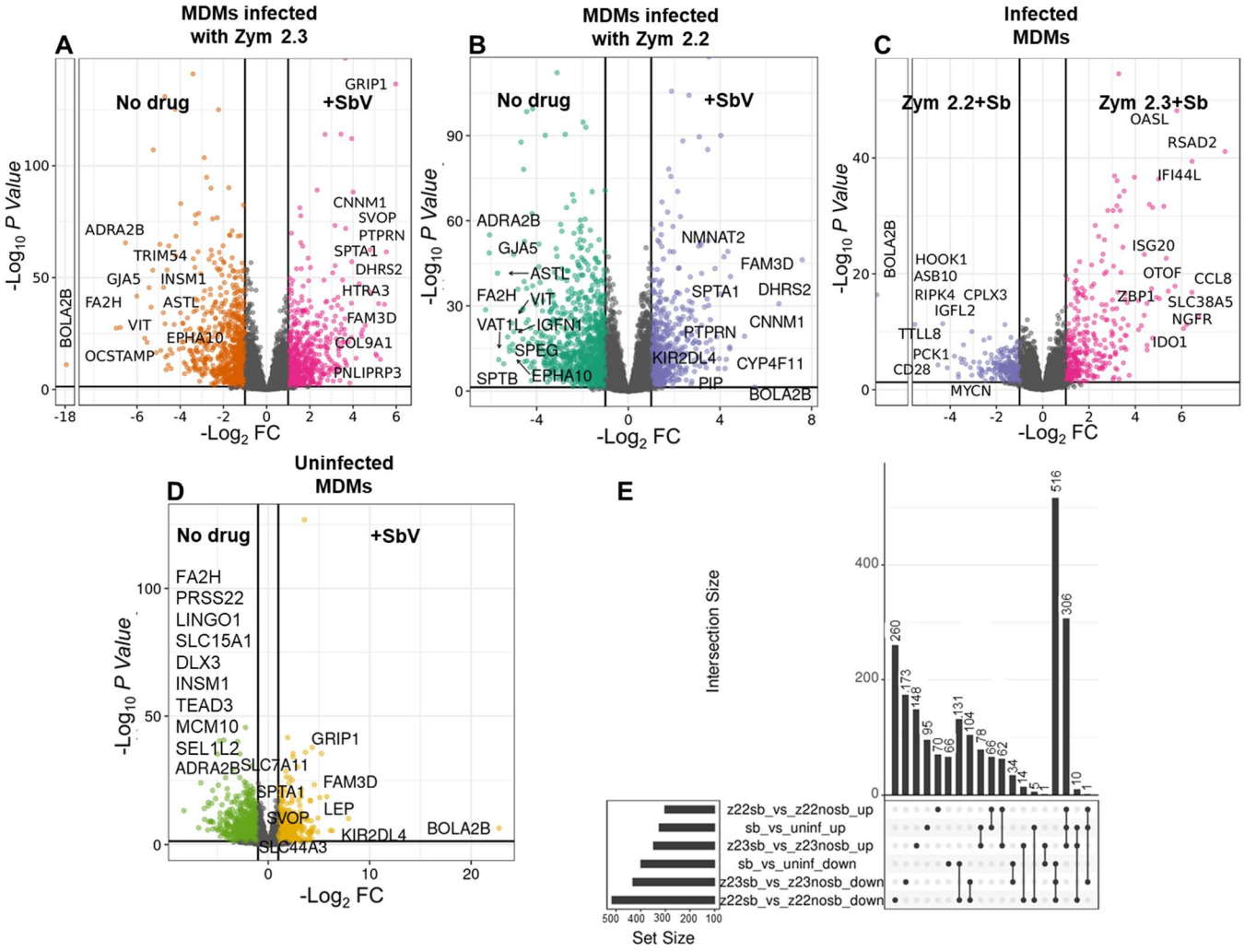

Figure 3. Differentially expressed genes in macrophages infected with zym 2.2 or zym 2.3, in the presence of SbV as meglumine antimoniate. A. Volcano plots visualizing comparisons of: **A**. MDMs infected with zym 2.3 strains + SbV vs. MDMs infected with zym 2.3 strains; **B.** MDMs infected with zym 2.2 strains + SbV vs. MDMs infected with zym 2.2 strains; **C**. MDMs infected with zym 2.3 strains + SbV vs. MDMs infected with zym 2.2 strains + SbV. **D.** MDMs uninfected + SbV vs. MDMs uninfected. **E**. UpSet plot summarizing the numbers of unique and shared upregulated and downregulated genes by infection with zym 2.3 and zym 2.2 strains, in the presence of SbV. The top 10 up or downregulated genes are labeled in panels **A-D**, and the color scheme is analogous to the one used in Fig.1. Data correspond to transcriptome analysis of MDMs from four healthy donors. Number of genes up- or down-regulated with fold change > 1 or < -1, and adj. $P < 0.05$.

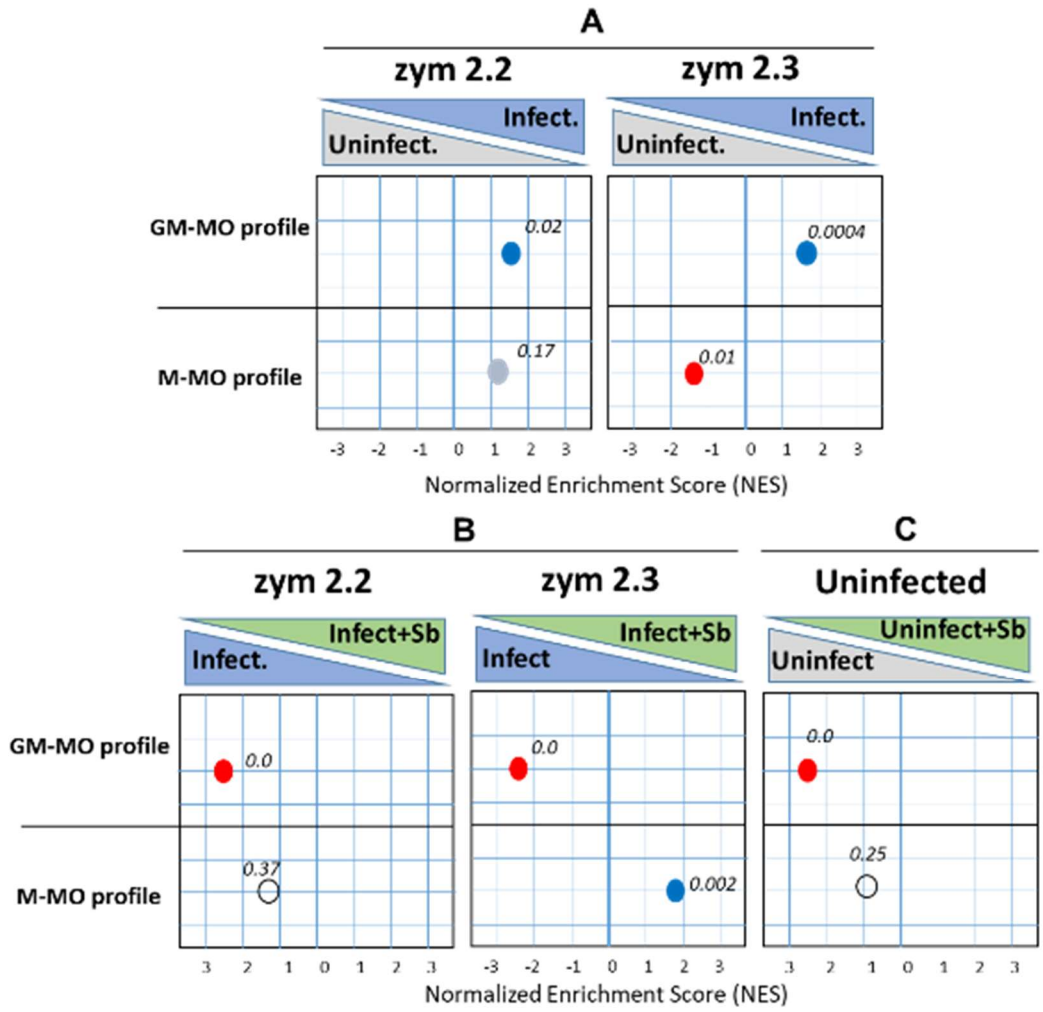

Figure 4. Gene set enrichment analysis of the global transcriptome profiles of macrophages exposed to different zymodeme infections and treatment conditions. GSEA on the ranked list of genes obtained from the reported comparison of the transcriptomes of M-MØ + LPS (anti-inflammatory MØ) versus GM-MØ (proinflammatory MØ) + LPS (GSE68061). GSEA of M-MØ- and GM-MØ-specific gene sets on the comparison of the **A.** macrophage infected with zym 2.3 and zym 2.2 strains vs uninfected, **B.** macrophage infected with zym 2.3 and zym 2.2 strains in presence of SbV vs infected macrophages, and **C.** uninfected macrophages plus SbV vs uninfected cells. Normalized Enrichment Score (NES) and False Discovery rate q value (FDRq) are indicated. Scores highlighted in red or blue indicate a significant negative or positive enrichment value for the each of the profiles.

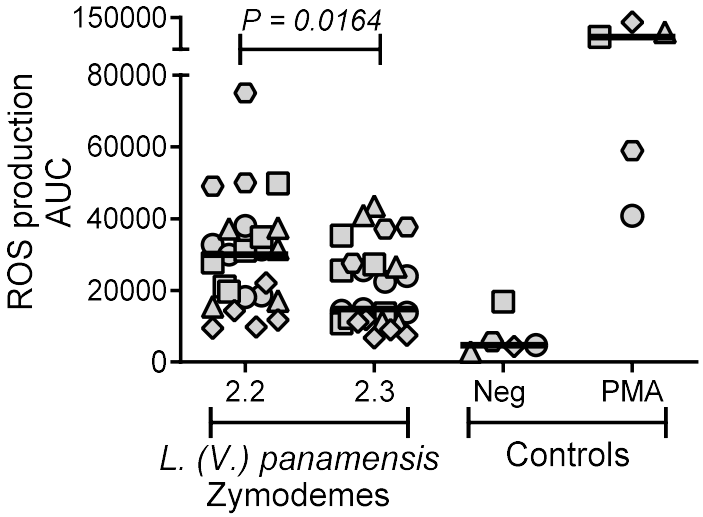

Figure 5. ROS production by human macrophages infected with clinical strains of *L. (V.) panamensis* pertaining to zym 2.3 and zym 2.2. Macrophages infected with zym 2.3 strains of *L. (V.) panamensis* (n = 6) compared with macrophages infected with zym 2.2 strains (n = 6), MDMs from healthy donors: n = 2 to 5 for each strain. Each geometric form corresponds to an individual donor. Horizontal bar represents median of AUC of ROS production, determined from relative light units (RLU).