The Journal of Infectious Diseases

BRIEF REPORT

Differential Expression of the Eicosanoid Pathway in Patients With Localized or Mucosal Cutaneous Leishmaniasis

Jaqueline França-Costa,[1] Bruno B. Andrade,[1,2] Ricardo Khouri,[1] Johan Van Weyenbergh,[1,6] Hayna Malta-Santos,[1,3] Claire da Silva Santos,[1] Cláudia I. Brodyskn,[1,3,4] Jackson M. Costa,[1] Aldina Barral,[1,3,4] Patrícia T. Bozza,[5] Viviane Boaventura,[1,3] and Valeria M. Borges[1,3]

[1]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), [2]Multinational Organization Network Sponsoring Translational and Epidemiological Research Initiative, Fundação José Silveira, and [3]Universidade Federal da Bahia, Salvador, [4]Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia, São Paulo, and [5]Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil; and [6]Department of Microbiology and Immunology, Rega Institute for Medical Research, University of Leuven, Belgium

Unfettered inflammation is thought to play critical role in the development of different clinical forms of tegumentary leishmaniasis. Eicosanoids are potent mediators of inflammation and tightly associated with modulation of immune responses. In this cross-sectional exploratory study, we addressed whether targets from the eicosanoid biosynthetic pathway, assessed by multiplexed expression assays in lesion biopsy and plasma specimens, could highlight a distinct biosignature in patients with mucocutaneous leishmaniasis (MCL) or localized cutaneous leishmaniasis (LCL). Differences in immunopathogenesis between MCL and LCL may result from an imbalance between prostaglandins and leukotrienes, which may serve as targets for future host-directed therapies.

Keywords. tegumentary leishmaniasis; inflammation; eicosanoids; prostaglandin; leukotrienes; biomarkers.

Tegumentary leishmaniasis is a vector-borne disease caused by *Leishmania* parasites and exhibits a wide spectrum of clinical presentations. The most common clinical form of the disease caused by *Leishmania braziliensis* is localized cutaneous leishmaniasis (LCL), characterized by ulcerated dermal lesions, which usually heal spontaneously [1]. A more severe form of this disease, mucocutaneous leishmaniasis (MCL), is observed in 3% of individuals with LCL [2]. Patients with MCL usually present with severe and progressive destruction of nasopharyngeal and/or laryngeal structures [2]. MCL lesions exhibit intense inflammation and tissue damage and, counterintuitively, very few parasites. Necrosis of mucosal tissue is associated with a strong T-cell–mediated response, reflected by an exacerbated delayed-type hypersensitivity (DTH) reaction to *Leishmania* antigens [3]. Possible mechanisms linked to increased disease severity in MCL are still unknown, but the lack of immune modulation leading to uncontrolled inflammation seems to be critically involved [3].

Eicosanoids have been described to regulate key aspects of the host immune responses during *Leishmania* infection [4]. Prostaglandin E_2 (PGE_2) has been shown to enhance parasite survival, whereas increased leukotriene B_4 (LTB_4) production leads to enhanced intracellular parasite killing by infected host cells [5, 6]. These findings suggest that the balance between prostanoids and leukotrienes may directly affect the capacity of the host to control *Leishmania* infection. However, whether this dichotomy in the expression of eicosanoids is relevant in the context of MCL remains unknown.

We performed a cross-sectional exploratory study in patients with MCL and those with LCL from an area of endemicity in Brazil, assessing circulating levels, as well as in situ RNA expression of mediators from the eicosanoid pathway. We identified a distinct biosignature of MCL, with a hallmark of decreased expression of enzymes and receptors of prostaglandins, compared with LCL. Moreover, plasma levels of PGE_2 and LTB_4 indicated that patients with MCL are prone to skew the eicosanoid balance toward leukotrienes, whereas individuals with LCL exhibit an enriched prostanoid signature. These distinct expression profiles have potential implications for the understanding of tegumentary leishmaniasis pathogenesis, which can lead to development of new host-directed therapies targeting the eicosanoid pathway.

PATIENTS AND METHODS

This study was approved by the institutional review board from Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (number 136/2007). All clinical investigations were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized.

The present study assessed age- and sex-matched patients with MCL (n = 13; male to female ratio, 1.4; mean age [± standard deviation {SD}], 59 ± 17 years) and those with LCL (n = 29; male to female ratio, 1.9; mean age [±SD], 34 ± 15 years) recruited at our reference clinic in Jiquiriçá, Brazil. The 2 groups were not significantly different with respect to age ($P = .894$) or sex distribution ($P = .921$). Individuals included in the present study were required to have no previous diagnosis of tegumentary leishmaniasis and to be treatment naive. For plasma analyses, we included samples from 43 healthy controls

Received 15 October 2015; accepted 11 November 2015; published online 17 November 2015.
Correspondence: V. M. Borges, Centro de Pesquisas Gonçalo Moniz, Rua Waldemar Falcão, 121, Candeal, Salvador, BA 40295-001, Brazil (vborges@bahia.fiocruz.br).

The Journal of Infectious Diseases® 2016;213:1143–7

DOI: 10.1093/infdis/jiv548

(matched by age and sex to the LCL and MCL groups) from a region of endemicity who had negative results of an anti-*Leishmania* DTH test. LCL and MCL diagnoses were confirmed by the presence of an ulcerated skin lesion or granulomatous mucosal lesion, respectively, in addition to at least 1 of the following: positive results of an anti-*Leishmania* DTH test, detection of anti-*Leishmania* antibody, or detection of *Leishmania* parasites in biopsy tissue specimens by either immunohistochemistry or qualitative polymerase chain reaction (PCR) assays. Patients with LCL exhibited a single or few ulcerated lesions for up to 2 months, while patients with MCL had symptoms for a prolonged period (mean disease duration [±SD], 10 ± 14 years) with mucosal lesions involving the nasal cavity (100%), pharynx (35%), and/or larynx (11%). Tissue samples from which we had high-quality messenger RNA (mRNA) were obtained from a subset of 4 patients with MCL and 7 patients with LCL. These patients were similar to their respective groups with regard to age and sex (data not shown). Nasal mucosal samples were obtained from turbinoplasty nasal surgery and performed under local anesthesia. All tissue specimens were obtained before treatment.

Total RNA was extracted from cryopreserved lesion biopsy specimens, using Trizol reagent (Invitrogen, Carlsbad, California), with an additional purification step using RNeasy columns (Qiagen, Venlo, Netherlands) as previously described [7]. nCounter analysis (NanoString Technologies, Seattle, Washington) was performed at the VIB MicroArray Facility (Leuven, Belgium), based on direct molecular bar coding of target RNA transcripts and digital detection [7]. The chosen targets were as follows: *PGES (*PGE synthase*)*, *PGDS* (PGD synthase), *PGD2R* (PGD 2 receptor), *PTGFR* (PGF receptor), *PTGS1* (COX-1), *PTGS2* (COX-2), *PLAS2G4A* (phospholipase S2G4A), *PLA2G6* (phospholipase 2G6), *LXA4R* (lipoxin A4 receptor), *PTGER1* (E-prostanoid receptor 1 [EP1]), *PTGR2* (EP2), *PTGER3 (*EP3*)*, *PTGER4* (EP4), *ALOX15* (arachidonate 15-lipoxygenase), *ALOX12* (arachidonate 12-lipoxygenase), and *ALOX5* (arachidonate 5-lipoxygenase). To account for differences in leukocyte infiltration between patient lesions, data were normalized for *CD45*, which encodes the pan-leukocyte marker CD45, detectable at the femtomolar range as previously reported [7].

Concentrations of PGE_2, PGD_2, $PGF_{2\alpha}$, LTB_4, and resolvin D1 (RvD1) were measured in cryopreserved ethylenediaminetetraacetic acid (EDTA)–treated plasma samples from all patients, using an enzyme-linked immunoassay (Cayman Chemical, Ann Harbor, Michigan).

Median values with interquartile ranges (IQRs) were used as measures of central tendency. For expression assays, the Mann–Whitney test was used to compare the variables. Plasma values were compared using the Kruskal–Wallis test with the Dunn multiple comparisons ad hoc test. Unsupervised 2-way hierarchical cluster analyses (Ward's method) with bootstrap were used to test whether patients with MCL and those with LCL can be grouped separately on the basis of simultaneous assessment of plasma eicosanoids. Two models of principal component analysis were used to test the contribution of PGE_2 levels to the power of the combined assessment of several eicosanoids to distinguish MCL from LCL cases. A P value of <.05 was considered statistically significant.

RESULTS AND DISCUSSION

To characterize the eicosanoid signaling pathways expressed in situ during MCL and LCL, we performed a comprehensive analysis of targeted RNA transcripts isolated from mucosal versus skin biopsy specimens. The target transcripts were represented within the context of an eicosanoid signaling pathway, using ingenuity pathway analysis (Figure 1*A*). Remarkably, patients with MCL exhibited substantial downmodulation in several genes from the prostaglandin pathway, compared with those with LCL (Figure 1*B*). Among all the genes examined, we found that *PGES*, *PTGER3*, *PGDS*, *PTGFR*, *PTGS1*, and *ALOX5* expression were significantly lower, whereas LXA4R expression was higher in MCL cases than in individuals with LCL (Figure 1*C*).

Interestingly, differences found in expression of constitutively expressed targets, such as *PTGS1* gene/COX-1, and for the inducible isoforms, such as *PTGS2* gene/COX-2, indicate that LCL and MCL activate distinct prostaglandins synthase pathways. PGE_2 acts through 4 distinct G protein-coupled receptors, EP1, EP2, EP3, and EP4 [8]. Once PGE_2 binds to different receptors, it can activate different signaling pathways inducing multiple, and sometime paradoxical, effector functions. Notably, it has been reported that *Leishmania major* infection upregulates EP1 and EP3 expression while downregulating EP2 and EP4 in vitro [9]. Our analyses suggested that differential expression of EP3 might be an important parameter related to the pathogenesis of MCL and LCL. Our exploratory findings warrant the design of additional studies that assess the role of EP receptor signaling in leishmaniasis.

We next tested whether the differences in gene expression observed in situ could be reflected in a distinct profile of plasma concentrations in patients with MCL and those with LCL. By quantifying plasma levels of PGE_2, PGD_2, and $PGF_{2\alpha}$, as well as LTB_4 and RvD1, we found that patients with LCL exhibited a distinct expression profile from those with MCL (Figure 2*A*). We observed that PGE_2 levels were significantly higher in LCL cases, compared with individuals with MCL (Figure 2*B*). Whether the augmented circulating levels of these prostanoids are directly related to the increased COX1/*PTGS1* expression in skin lesions observed in patients with LCL deserves future clarification. Importantly, compared with values detected in healthy controls, PGD_2, $PGF_{2\alpha}$, LTB_4, and RvD1 levels were significantly lower in patients with LCL (Figure 2*B*). Conversely, concentrations of all eicosanoids except $PGF_{2\alpha}$ were undistinguishable

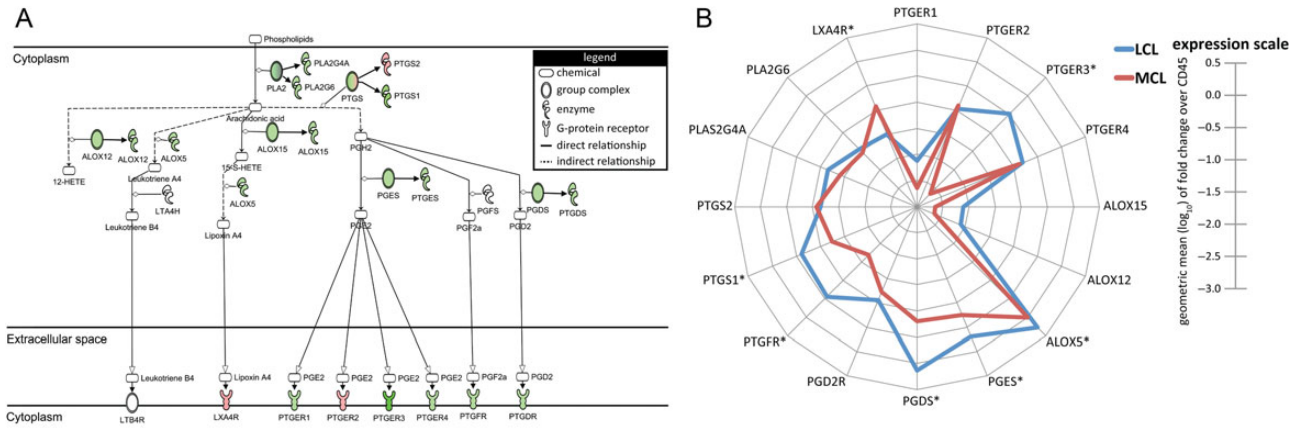

Figure 1. Differential expression of selected genes of eicosanoid pathways in skin or mucosal lesions from patients with tegumentary leishmaniasis. Total RNA was extracted from lesion biopsy specimens obtained from 7 patients with localized cutaneous leishmaniasis (LCL) and 4 with mucocutaneous leishmaniasis (MCL). Indicated messenger RNA transcripts of host-specific cellular genes were quantified by nCounter (Nanostring), including the pan-leukocyte gene CD45, for normalization of immune infiltration into tissues. *A*, The targeted genes were represented within the context of an eicosanoid signaling pathway, using Ingenuity Pathway Analysis. Red and green colors infer higher or lower gene expression in patients with MCL, relative to that in patients with LCL, respectively. *B*, A representative profile of geometric mean values (log_{10} transformed) for indicated genes is displayed for each clinical group. *C*, Scatterplots of gene expression relative to CD45 are shown. Lines represent median values and interquartile ranges. Data were compared using the Mann–Whitney test. $^{*}P < .05$.

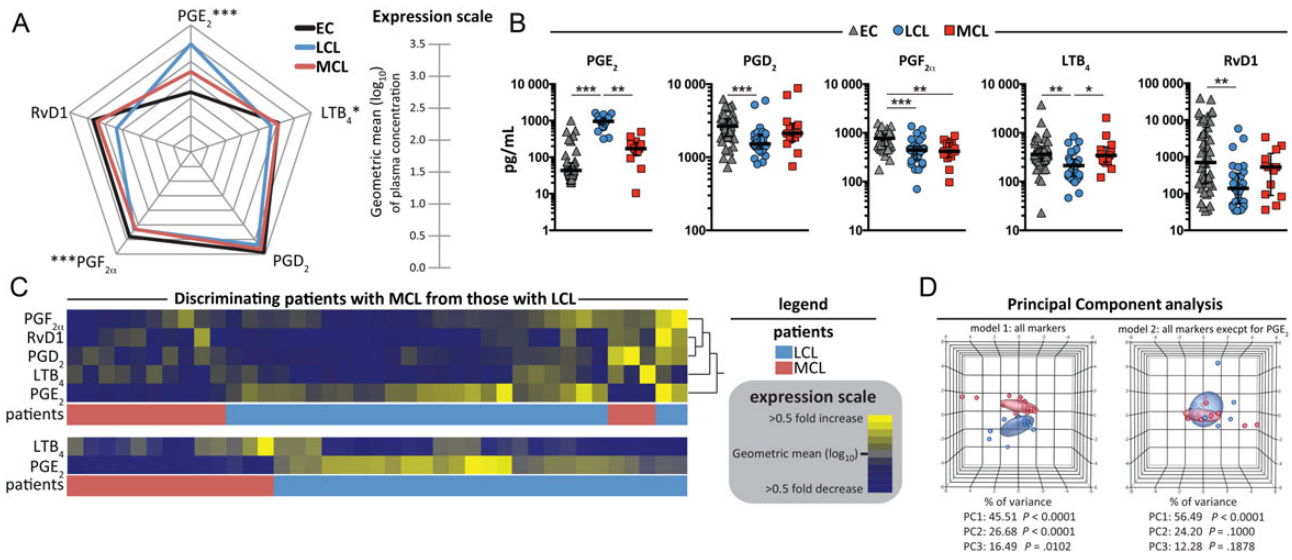

Figure 2. Plasma concentrations of eicosanoids in patients with localized cutaneous leishmaniasis (LCL) or mucosal cutaneous leishmaniasis (MCL). *A*, Plasma levels of COX-2–derived prostanoids prostaglandin E_2 (PGE_2), PGD_2, and $PGF_{2\alpha}$, as well as 5-LO–derived lipid mediators leukotriene B_4 (LTB_4) and resolvin D1 (RvD1), were compared between 29 patients with LCL or 13 with MCL, as well as 43 healthy controls from an area of endemicity (EC). Data were compared using the Kruskal–Wallis test with the Dunn multiple comparisons ad hoc test. Lines represent median values and interquartile ranges. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. *B*, Univariate analyzes with scatterplots of the comparisons are shown. *C*, A hierarchical clustering analysis (Ward's method) was used to test whether the overall expression profile of all the lipid mediators (upper panel) or just PGE_2 and LTB_4 (lower panel) in plasma could distinguish MCL from LCL cases. *D*, Two principal component (PC) analysis models were used to examine the contribution of PGE_2 in explaining the differences observed between the LCL and MCL study groups.

between patients with MCL and controls (Figure 2*B*). Noteworthy, levels of LTB_4 were >1 log higher in patients with MCL than in LCL cases (Figure 2*B*). Leukotrienes are highly bioactive, and minor differences in plasma measurements could reflect major differences in inflammation, as observed in other disease models [10]. These results indicate that a downmodulatory effect may be more relevant in LCL than in MCL, compared with healthy donors. Thus, systemic mediators observed in LCL and MCL may be useful as biomarkers of active disease.

Together, these observations led us to hypothesize that a balance in the circulating levels of lipid mediators is associated with the differential inflammatory status observed in MCL or LCL. In this scenario, prostaglandins derived from cyclooxygenases would prevail over lipoxygenase-derived products in patients with LCL, compared with those with MCL. To test this hypothesis, we performed an unsupervised hierarchical cluster analysis in which plasma values of all the eicosanoids measured in patients with MCL or LCL were inputted. We confirmed that simultaneous assessment of key prostaglandins, LTB_4 and RvD1, could successfully segregate the different clinical groups evaluated (Figure 2*C*).

RvD1, an important specialized proresolving mediator, is endogenously generated during the spontaneous resolution phase in many models of acute and chronic inflammation diseases [11]. Counterintuitively at a first glance, although our data reveal that there is no statistically significant difference for RvD1 plasma levels between patients with LCL and those with MCL, we noticed a trend of RvD1 median levels to be approximately 1.2 log decreased during LCL, compared with MCL (Figure 2*B*). Whether RvD1 participates in the control and resolution of inflammation or promotion of parasite survival in patients with tegumentary leishmaniasis needs to be further investigated.

Notably, to our knowledge, this is the first study to demonstrate increased levels of prostanoids in plasma specimens from patients with tegumentary leishmaniasis. Interestingly, we found that $PGF_{2\alpha}$ was in general reduced in patients with tegumentary leishmaniasis, compared with controls (Figure 2*B*). Recent studies from our group reported that $PGF_{2\alpha}$ is uniquely involved in the cellular metabolism of *Leishmania* species and its immune evasion capacity in murine models [12, 13]. Modulation of $PGF_{2\alpha}$ production could be a potential mechanism by which the host restricts a key mediator for promotion of parasite growth.

Strikingly, additional hierarchical clustering analyses confirmed that patients with MCL and those with LCL could be better separated when data on only 2 eicosanoids, PGE_2 and LTB_4, were considered (Figure 2*C*). The balance between these eicosanoids has been described to determine clinical outcomes in other diseases [10]. Two models of principal component analysis supported the idea that differential expression of PGE_2 in plasma is probably the most important parameter leading to distinction between patients with MCL and those with LCL (Figure 2*D*). Importantly, the PGE_2/LTB_4 ratio was >8-fold higher in patients with LCL than in those with MCL (median values, 5.3 [IQR, 3.5–6.4] vs 0.6 [IQR, 0.1–0.8]; $P < .0001$). Thus, circulating levels of PGE_2 and LTB_4 could be tested as potential biomarkers of mucosal involvement in tegumentary leishmaniasis. Considering that MCL cases exhibit longer periods with disease prior to diagnosis and that this disease form may progress from localized lesions, it is possible that our results may be affected by illness duration. Prospective studies focused on early detection of MCL may help clarify this issue. In addition, differences in the expression profile of these biomarkers may reflect distinctions in the infiltration of leukocytes in the lesions. Although MCL and LCL exhibit, in general, cellular infiltrates enriched for mononuclear cells, we have previously shown an important role for neutrophils contributing to inflammation in MCL [14]. Cellular analyses using flow cytometry could be performed to extensively phenotype cellular subsets recruited to tegumentary lesions, thus elucidating associations between the leukocyte infiltrate and the differential eicosanoid expression described here. A limitation of the present study was the lack of access to skin/mucosal biopsy specimens from healthy controls for comparison with those from patients with leishmaniasis. In addition, we could not test correlations between eicosanoid expression and parasite burden in the lesions, because the parasite quantification was very low and the sensitivity of the histological technique was insufficient to provide reliable quantitative values in such a small sample set of individuals from whom we had in situ data. Regardless, our data indicate that eicosanoids pathways, and PGE_2 in particular, may be explored as novel targets for therapeutic interventions of tegumentary leishmaniasis.

Notes

Acknowledgments. We thank Andrezza Souza for technical and logistics support.

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support. This work was supported by the Fundação de Amparo à Pesquisa do Estado da Bahia (grants RED0018/2013 and PET0036/2013 to V. M. B.), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant 478480/2013-0 to V. M. B.; fellowship to H. M.-S.; V. M. B., C. I. B., P. T. B., and A. B. are employees), the Ciências sem Fronteiras/CNPq Pesquisador Visitante Especial (400312/2012-3 to J. V. W., A. B., and R. K.) and the CsF/CNPq Bolsa Jovem Talentos (405502/2013-3 to R. K.).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Silveira FT, Lainson R, Corbett CE. Clinical and immunopathological spectrum of American cutaneous leishmaniasis with special reference to the disease in Amazonian Brazil: a review. Mem Inst Oswaldo Cruz **2004**; 99:239–51.
2. Dutra WO, de Faria DR, Lima Machado PR, et al. Immunoregulatory and effector activities in human cutaneous and mucosal leishmaniasis: understanding mechanisms of pathology. Drug Dev Res **2011**; 72:430–6.

3. Bacellar O, Lessa H, Schriefer A, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. Infect Immun **2002**; 70:6734–40.
4. Daugschies A, Joachim A. Eicosanoids in parasites and parasitic infections. Adv Parasitol **2000**; 46:181–240.
5. Lonardoni MV, Barbieri CL, Russo M, Jancar S. Modulation of leishmania (L.) amazonensis growth in cultured mouse macrophages by prostaglandins and platelet activating factor. Mediators Inflamm **1994**; 3:137–41.
6. Morato CI, da Silva IA Jr, Borges AF, et al. Essential role of leukotriene B4 on Leishmania (Viannia) braziliensis killing by human macrophages. Microbes Infect **2014**; 16:945–53.
7. Franca-Costa J, Van Weyenbergh J, Boaventura VS, et al. Arginase I, polyamine, and prostaglandin E2 pathways suppress the inflammatory response and contribute to diffuse cutaneous leishmaniasis. J Infect Dis **2015**; 211:426–35.
8. Kawahara K, Hohjoh H, Inazumi T, Tsuchiya S, Sugimoto Y. Prostaglandin E2-induced inflammation: relevance of prostaglandin E receptors. Biochim Biophys Acta **2015**; 1851:414–21.
9. Penke LR, Sudan R, Sathishkumar S, Saha B. Prostaglandin E(2) receptors have differential effects on Leishmania major infection. Parasite Immunol **2013**; 35:51–4.
10. Mayer-Barber KD, Andrade BB, Oland SD, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature **2014**; 511:99–103.
11. Buckley CD, Gilroy DW, Serhan CN. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. Immunity **2014**; 40:315–27.
12. Araujo-Santos T, Rodriguez NE, Moura-Pontes S, et al. Role of prostaglandin F2alpha production in lipid bodies from Leishmania infantum chagasi: insights on virulence. J Infect Dis **2014**; 210:1951–61.
13. Alves-Ferreira EV, Toledo JS, De Oliveira AH, et al. Differential gene expression and infection profiles of cutaneous and mucosal leishmania braziliensis isolates from the same patient. PLoS Negl Trop Dis **2015**; 9:e0004018.
14. Boaventura VS, Santos CS, Cardoso CR, et al. Human mucosal leishmaniasis: neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. Eur J Immunol **2010**; 40:2830–6.