Polyunsaturated fatty acid metabolites: biosynthesis in *Leishmania* and role in parasite/host interaction[S]

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Abstract **Inside the human host, *Leishmania* infection starts with phagocytosis of infective promastigotes by macrophages. In order to survive, *Leishmania* has developed several strategies to manipulate macrophage functions. Among these strategies, *Leishmania* as a source of bioactive lipids has been poorly explored. Herein, we assessed the biosynthesis of polyunsaturated fatty acid metabolites by infective and noninfective stages of *Leishmania* and further explored the role of these metabolites in macrophage polarization. The concentration of docosahexaenoic acid metabolites, precursors of proresolving lipid mediators, was increased in the infective stage of the parasite compared with the noninfective stage, and cytochrome P450-like proteins were shown to be implicated in the biosynthesis of these metabolites. The treatment of macrophages with lipids extracted from the infective forms of the parasite led to M2 macrophage polarization and blocked the differentiation into the M1 phenotype induced by IFN-γ. In conclusion, *Leishmania* polyunsaturated fatty acid metabolites, produced by cytochrome P450-like protein activity, are implicated in parasite/host interactions by promoting the polarization of macrophages into a proresolving M2 phenotype.**—Paloque, L., T. Perez-Berezo, A. Abot, J. Dalloux-Chioccioli, S. Bourgeade-Delmas, P. Le Faouder, J. Pujo, M-A.Teste, J-M. François, N. H. Schebb, M. Mainka, C. Rolland, C. Blanpied, G. Dietrich, J. Bertrand-Michel, C. Deraison, A. Valentin, and N. Cenac. **Polyunsaturated fatty acid metabolites: biosynthesis in *Leishmania* and role in parasite/host interaction. *J. Lipid Res.* 2019.** 60: **636–647.**

Supplementary key words cytochrome P450 • infection • macrophages • ω-3 • metabolism • arachidonic acid • eicosanoids

Leishmaniasis is a plague affecting millions of people around the world, most notably in developing countries but also in the Mediterranean. It is the second-most lethal parasitic infection worldwide, with more than 20,000 annual deaths (http://www.who.int/leishmaniasis/en). This disease is supported by the intracellular development of a *protozoan* parasite belonging to the *Leishmania* genus. Metacyclic promastigotes are transmitted by the *Phlebotominae* sandfly during a blood meal on mammals, where they infect macrophages prior to differentiating into amastigote forms and proliferating inside the host cell. Procyclic promastigotes differentiate into metacyclic promastigotes during metacyclogenesis, a process that is triggered by an environmental pH decrease from neutral to acidic pH inside the sandfly vector (1, 2). Metacyclogenesis involves important morphological and biochemical modifications, and approximately 300 genes have been described to be specifically regulated according to the parasite stage (3–6). Macrophages are the primary replication site for *Leishmania* and the main effector cells to fight it (7). *Leishmania* infection leads to the development of macrophages that overexpress anti-inflammatory molecules such as interleukin (IL) 10 and transforming growth factor β and the induction of proinflammatory cytokine production such as TNF-α, IL-1β, and IL-6 (8). M2 macrophages control disease severity and protect the host

This work was supported by grants from Région Midi-Pyrénées (N.C.) and European Commission Marie Curie Action EPIMACASE Grant IOF-627487 (C.D.).

Manuscript received 19 December 2018 and in revised form 8 January 2019.

Published, JLR Papers in Press, January 9, 2019
DOI https://doi.org/10.1194/jlr.M091736

Abbreviations: AA, arachidonic acid; Arg1, arginase; BMDM, bone marrow-derived macrophage; CYP, cytochrome P450; DiHDPE, dihydroxydocosapentaenoic acid; EpDPE, epoxydocosapentaenoic acid; EpETrE, epoxyeicosatrienoic acid; HDoHE, hydroxydocosahexaenoic acid; IL, interleukin; LA, linoleic acid; MeOH, methanol; NDGA, nordihydroguaiaretic acid; PDx, protectin Dx; PG, prostaglandin; qPCR, quantitative PCR; RvD, resolvin D; SPM, specialized proresolving mediator; 5-oxo-ETE, 5-oxoeicosatetraenoic acid; 7-MaR1, 7-maresin 1; 17-ODYA, 17-octadecynoic acid.

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[S] The online version of this article (available at http://www.jlr.org) contains a supplement.

from the detrimental effect of an excessive type 1 T helper cell response, making "symbiotic" survival between the host and parasite more likely (7, 9, 10). In order to survive in the macrophage, *Leishmania* has to prevent or inhibit a variety of intracellular mechanisms of parasite elimination. Among the strategies developed by the parasite for manipulating macrophage functions (7, 9, 10), *Leishmania* as a source of bioactive lipids has been poorly explored (11), despite the fact that very close protozoan pathogens, *Trypanosoma sp.*, are known to produce a number of oxylipins (12). Herein, we explore the biosynthesis of PUFA metabolites in *Leishmania infantum* during infectivity acquisition and their putative role in the interaction with macrophages that trigger a minimal proinflammatory response in the host for the benefit of the parasite.

METHODS

Ethics statement

Male C57Bl/6J mice (6–8 weeks; Janvier Labs, Saint-Quentin-Fallavier, France) were used to produce primary cultures of macrophages. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the European Council and were approved by the Animal Care and Ethics Committee of US006/CREFE (CEEA-122).

Leishmania culture conditions

The *Leishmania* species used in this study was *Leishmania infantum* (MHOM/MA/67/ITMAP-263). Procyclic promastigotes were cultured in the log phase in RPMI 1640 medium (VWR, Fontenay-sous-Bois, France) supplemented with 10% FCS (VWR), 2 mM L-glutamine (Thermo Fisher Scientific, Illkirch-Graffenstaden, France), 25 mM HEPES (Thermo Fisher Scientific), 100 U/ml penicillin (Thermo Fisher Scientific), 100 µg/ml streptomycin (Thermo Fisher Scientific), and 50 µg/ml geneticin (Sigma-Aldrich, Lyon, France), pH 7.7, at 24°C.

Cell culture experiments

CHO cells (CCL-61; ATCC, Molsheim, France) were maintained in Ham's F12 Nutrient Mixture (Thermo Fisher Scientific) supplemented with 5% FCS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in 5% CO_2.

Bone marrows were isolated from murine femurs. Cells were cultured in RPMI 1640 supplemented with 20% FBS (VWR), 100 U/ml penicillin, and 100 µg/ml streptomycin and supplemented with 20 ng/ml mouse macrophage colony-stimulating factor (R&D Systems, Abingdon, UK) for 7 days.

Induction of *Leishmania* metacyclogenesis

Procyclic promastigotes in the log phase were centrifuged at 900 *g* for 10 min. Cells were then suspended in RPMI 1640 complete medium (pH 5.4) and incubated at 24°C for 24 h to obtain metacyclic promastigotes.

Inhibition of lipid metabolism by different inhibitors

Metacyclic promastigotes (2×10^6 promastigotes/ml in RPMI 1640 complete medium, pH 5.4) were treated with either 100 µM nordihydroguaiaretic acid (NDGA; Alfa Aesar, Karlsruhe, Germany), 100 µM 17-octadecynoic acid (17-ODYA; Cayman Chemicals, Ann Arbor, MI), 10 µM clotrimazole (Sigma-Aldrich), or 10 µM tioconazole (Sigma-Aldrich) for 24 h at 24°C.

Leishmania oxylipin extraction

Parasites were harvested by centrifugation at 900 *g* for 10 min and then washed three times with PBS (Sigma-Aldrich). Pellets of 1×10^8 parasites in 200 µl PBS were snap-frozen and stored in liquid nitrogen prior to lipid extraction. Thawed pellets in PBS were transferred to lysing matrix tubes containing 5 µl internal standard mixture [lipoxin A4-d5, leukotriene B4-d4, and 5-HETE-d8 at 400 ng/ml in methanol (MeOH); Cayman Chemicals] and immediately crushed with a FastPrep-24 Instrument (MP Biomedical, Santa Ana, CA). After two crush cycles (6.5 m/s, 30 s), 10 µl suspension were withdrawn for protein quantification, and 0.3 ml cold MeOH were added. Samples were centrifuged thereafter at 1,016 *g* for 15 min at 4°C, and the resulting supernatants were submitted to solid-phase extraction of lipids using HRX-50 mg 96-well plates (Macherey-Nagel, Hoerd, France). In brief, plates were conditioned with 2 ml MeOH and 2 ml water-MeOH (90:10; v/v). Samples were loaded at flow rate of about 1 drop per 2 s and, after complete loading, columns were washed with 2 ml water-MeOH (90:10; v/v). The columns were dried thereafter under aspiration, and lipids were eluted with 2 ml MeOH. Solvent was evaporated under N_2, and samples were resuspended in 10 µl MeOH and stored at 80°C for LC/MS/MS analysis or macrophage treatments.

LC/MS/MS measurements of proinflammatory and proresolving lipids

The quantification of 6-keto-prostaglandin (PG) F1α, thromboxane B2, PGE_2, PGA_1, 8-iso-PGA_2, PGE_3, 15d-PGJ_2, $PGF_{2\alpha}$, PGD_2, lipoxin A4, lipoxin B4, resolvin D (RvD) 1, RvD2, 7-maresin 1 (7-MaR1), leukotriene B4, leukotriene B5, protectin Dx (PDx), 18-hydroxyeicosapentaenoic acid, 5,6-DiHETE, 15-HETE, 12-HETE, 8-HETE, 5-HETE, 13-HODE, 9-HODE, 17-hydroxydocosahexaenoic acid (HDoHE), 14-HDoHE, 14(15)-epoxyeicosatrienoic acid (EpETrE), 11(12)-EpETrE, 8(9)-EpETrE, 5(6)-EpETrE, and 5-oxoeicosatetraenoic acid (5-oxo-ETE) was performed at different stages of the life cycle of *L. infantum* and in stimulated macrophages. To simultaneously separate the 32 lipids of interest and the 3 deuterated internal standards, LC/MS/MS analysis was performed as previously described (13) on an Agilent LC1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Les Ulis, France) equipped with electrospray ionization operating in the negative mode. Reverse-phase ultra-high-performance liquid chromatography was performed using a ZORBAX SB-C18 column (inner diameter: 2.1 mm; length: 50 mm; particle size: 1.8 µm; Agilent Technologies) with a gradient elution.

LC/MS/MS measurements of epoxy and hydroxyl PUFAs

The detection of oxylipins was adapted from Rund et al. (14) and performed using an Agilent 6460 triple quadrupole mass spectrometer equipped with an Agilent 1290 Infinity HPLC system and ZORBAX Eclipse Plus C18 column (inner diameter: 2.1 mm; length: 150 mm; particle size: 1.8 µm). For HPLC separation, the mobile phase A was 0.1% acetic acid in water (v/v), and the mobile phase B was acetonitrile, MeOH, and acetic acid (800/150/1; v/v/v). Oxylipins were separated using an optimized 32.2-min gradient of 0.0–1.0 min, 26% B; 1.0–1.5 min, 35% B; 1.5–8.0 min, 49.5% B; 8.0–16.0 min, 59.8% B; 16.0–25.1 min, 72% B; 25.1–27.6 min, 98% B; 27.6–29.7 min, 98% B; 29.7–29.8 min, 26% B; and 29.8–32.2 min, 26% B. The flow rate was set at 0.35 ml/min, and the column temperature was set at 40°C. Electrospray ionization was used in the negative mode. The MS parameters were as follows: source temperature, 325°C; desolvatation temperature, 350°C; cone gas flow, 10 l/min; desolvatation gas flow, 12 l/min; nebulizer pressure, 30 psi; capillary voltage, 3,000 V; and nozzle voltage, 2,000 V. Data were acquired in dynamic multiple reaction monitoring mode with optimized conditions (ion optics and collision energy). Peak

detection and integration analysis were done using MassHunter quantitative analysis software (Agilent Technologies).

Leishmania fatty acid extraction

Parasites were harvested by centrifugation at 900 *g* for 10 min and then washed three times with PBS (Sigma-Aldrich). Pellets of 1×10^8 parasites in 200 µl PBS were snap-frozen and stored in liquid nitrogen prior to lipid extraction. Thawed pellets in PBS were transferred to lysing matrix tubes with 1 ml MeOH-EGTA (5 mM). After homogenization, 75% of the sample was extracted according to Bligh and Dyer (15) in dichloromethane-MeOH-water (2.5:2.5:2.1; v/v/v) in the presence of the internal standard heptadecanoate acid (2 µg) for the free fatty acid profiling. The lipid extract was then directly methylated in 1 ml 14% boron trifluoride MeOH solution (Sigma-Aldrich) and 1 ml heptane at room temperature for 10 min. After the addition of 1 ml water to the crude, methylated free fatty acids were extracted with 3 ml heptane, evaporated to dryness, and dissolved in 20 µl ethyl acetate. In addition, 25% of the crushed parasite was extracted in the presence of 2 µg glyceryl triheptadecanoate to profile the total fatty acids. The lipid extract was hydrolyzed in potassium hydroxide (0.5 M in MeOH) at 50°C for 30 min and transmethylated in 1 ml 14% boron trifluoride MeOH solution and 1 ml heptane at 80°C for 1 h. After the addition of 1 ml water to the crude, methylated total fatty acids were extracted with 3 ml heptane, evaporated to dryness, and dissolved in 20 µl ethyl acetate.

GC with flame ionization detection of total and free fatty acids

Methylated free or total fatty acids (1 µl) were analyzed by GC/LC (16) on a Clarus 600 Perkin Elmer system using FAMEWAX RESTEK fused silica capillary columns (inner diameter: 0.32 mm; length: 30 m; film thickness: 0.25 µm). The oven temperature was programmed from 110 to 220°C at a rate of 2°C/min, and the carrier gas was hydrogen (0.5 bar). The injector and detector were set at 225 and 245°C, respectively. Peak detection and integration analysis were done using Azur software.

Leishmania RNA extraction

RNA extraction from 2×10^7 procyclic promastigotes and metacyclic promastigotes was performed with the TRIzol + RNA extraction kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

RT-qPCR

RNA (1 µg) was reverse-transcribed using the SuperScript First-Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer's recommendations. Transcriptional levels of specific mRNAs were determined by quantitative PCR (qPCR) using the SYBR Green PCR Master Mix and performed on a LightCycler 480 (Roche Diagnostics, Meylan, France). Primers (Eurofins Genomics, Les Ullis, France) used for qPCR are listed in **Table 1** for cytochrome P450 (CYP). Primers (Eurofins Genomics) used to characterize macrophage phenotype were edited by Accarias et al. (17). Expression ratios were calculated according to the expression level of the housekeeping gene *LinJ.36.0990* coding for 40S ribosomal protein S18 (18) and *RLP19* in the parasite and macrophage, respectively.

Leishmania CYP cloning

DNA was extracted from 2×10^7 procyclic promastigotes with the Wizard Genomic DNA Purification Kit (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions for CYP2 and CYP3. *L. infantum* JPCM5 genome chromosome 27 (CYP1) was purchased from CliniSciences (Nanterre, France) cloned in the pUC57 vector with XbaI/NotI used as the cloning site. Primers (Eurofins Genomics) used for *CYP* cloning were 5′-CACCTTCTCAGCCTTGGGTTCCA-3′ and 5′-ATGGCAGCGTTTAGTCGTCTCC-3′ for CYP2 and 5′-CCCGCGTACGGCGGCTTCTTT-3′ and 5′-ATGGCCCCCACTGTCTCGCCA-3′ for CYP3. CYP-amplified inserts were subcloned in pCI-Neo (Promega) between XbaI and NotI restriction sites downstream to the constitutive promoter cytomegalovirus. Plasmid construction was verified by sequence alignment with a reference sequence from GenBank.

Transfection assay in the CHO cell line

Twenty-four hours before transfection, CHO cells were seeded in 24-well plates at 50% confluence in Opti-MEM medium. Transfections were carried out using Gene Juice transfection reagent (Merck, Kenilworth, NJ) with 500 ng of empty pCI-neo plasmid (Promega) containing *CYP1*, *CYP2*, or *CYP3*. Following an overnight incubation, culture medium was removed and replaced. Twenty-four hours thereafter cells were placed in HBSS (200 µl) containing vehicle (HBSS), 10 µM arachidonic acid (AA), or 10 µM DHA for 10 min at room temperature. Cells and HBSS were then collected for lipid quantification.

Bone marrow macrophage stimulation

Macrophage progenitors were isolated from the femurs of C57BL6 WT mice. Cells were differentiated and maintained in culture with 20 ng/ml macrophage colony-stimulating factor for 7 days before use. Bone marrow-derived monocytes were stimulated for 24 h with oxylipin extracted from procyclic or metacyclic promastigotes (10 µl in MeOH). For some experiments, monocytes were also incubated with oxylipin extracted from *Leishmania* or vehicle (MeOH) and costimulated with 20 ng/ml IFN for 24 h. Cells were collected in HBSS and submitted to lipidomic analysis. For transcriptional analysis, cells were lysed in TRIzol, and mRNA was collected according to the manufacturer's instructions.

Statistical analysis

Data are presented as means ± SEMs. GraphPad Prism version 6.0 (GraphPad, San Diego, CA) was used for statistical analysis. Multiple comparisons within groups were performed by the Kruskal-Wallis test followed by Dunn's post hoc test. Comparisons among groups were performed by the Mann-Whitney test. Statistical significance was accepted at $P < 0.05$.

TABLE 1. Primers for RT-qPCR

Gene ID on GeneDB[a]			5′-3′
LinJ.36.0990	40S S18	Forward	GCGCAAGGTGCCGTTCGCG
		Reverse	GCTCCACGTCGATCCCGGC
LinJ.27.0090	CYP1	Forward	GCCTGCTGCTGTCGCATGCG
		Reverse	TTCGCGTCAACCGCGTCGAGC
LinJ.30.3610	CYP2	Forward	CGGACGCTGAGCCGCACTG
		Reverse	GCCATGGCGTGGTCCGACG
LinJ.34.3110	CYP3	Forward	GCGAAGAAGGCGAGGCGCTG
		Reverse	AGGTAGCCGAGCTCCTGGCC

[a]See http://www.genedb.org.

RESULTS

PUFA metabolite production by *L. infantum* varies during infectivity acquisition

Free PUFA metabolite production during metacyclogenesis was assessed by LC/MS/MS in procyclic promastigotes (noninfective stage) and metacyclic promastigotes (infective stage) obtained in vitro by preconditioning procyclic promastigotes at acidic pH 5.4 for 24 h (19). Of the 32 PUFA metabolites assessed, 10 were detected and quantified in both the procyclic and metacyclic stages of *L. infantum* (**Fig. 1A**). These metabolites were derived from linoleic

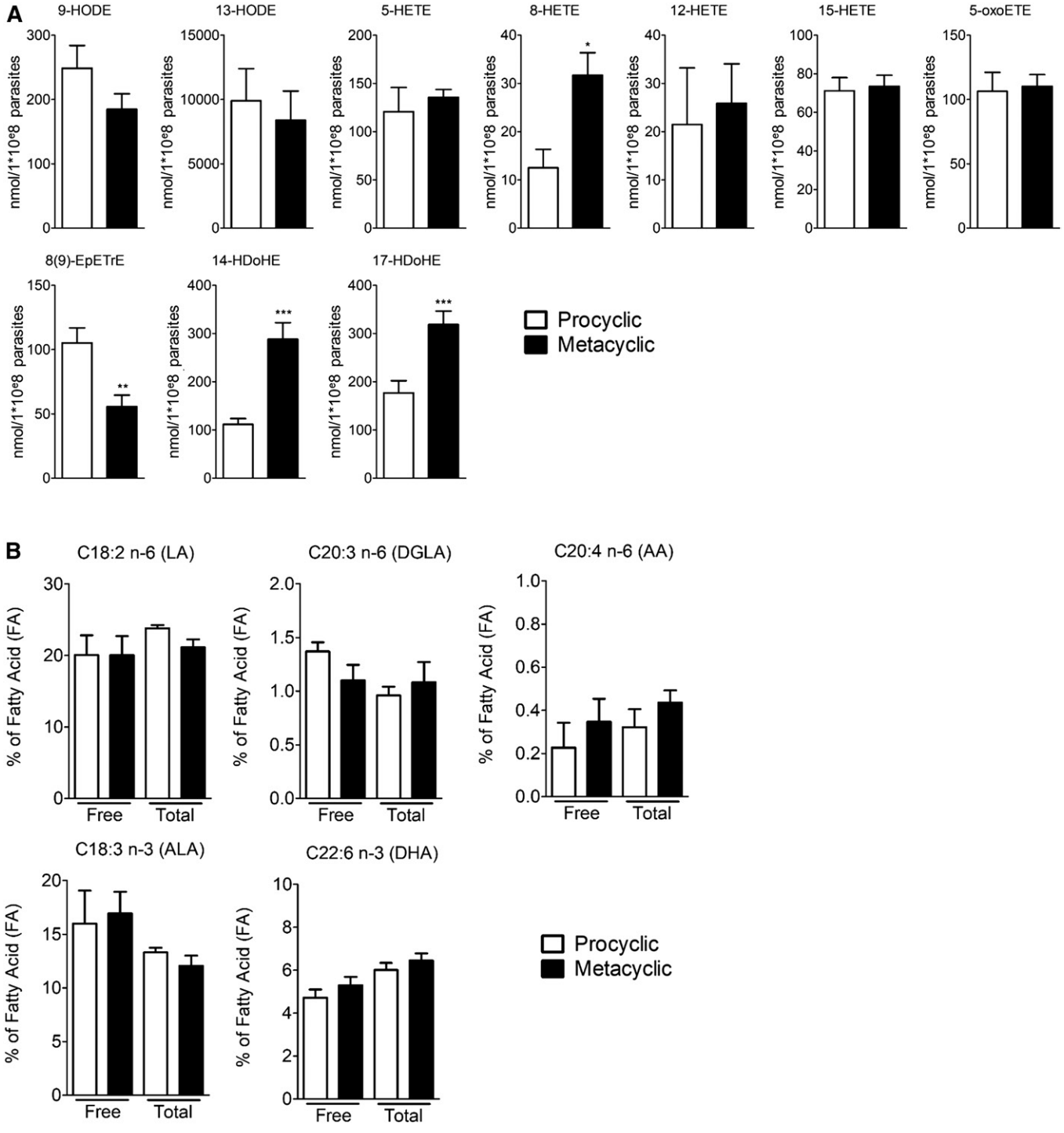


Fig. 1. PUFA metabolite production by *L. infantum* varies during infectivity acquisition. A: Production of PUFA metabolites assessed by LC/MS/MS on *Leishmania* lipid extracts from procyclic and metacyclic promastigotes. Data shown are from six experiments conducted in triplicate; means ± SEMs are shown ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the procyclic group. B: Percentage of main PUFAs (LA, ALA, DGLA, AA, and DHA) in free and total fatty acids assessed by GC with flame ionization detection on *Leishmania* lipid extracts from procyclic and metacyclic promastigotes. Data shown are from three experiments conducted in triplicate; means ± SEMs are shown ($n = 3$). ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid.

acid (LA) (9- and 13-HODE), AA [8(9)-EpETrE, 5-HETE, 8-HETE, 12-HETE, 15-HETE, and 5-oxo-ETE], and DHA (14- and 17-HDoHE). The infective form (metacyclic promastigote stage) of the parasite showed an increased concentration of 8-HETE, 14-HDoHE, and 17-HDoHE compared with the noninfective form (Fig. 1A). On the contrary, the quantity of 9-HODE and 8(9)-EpETrE was reduced during metacyclogenesis, as shown by the decreased concentration of these metabolites in the metacyclic compared with procyclic stage of the parasite (Fig. 1A). As the quantified metabolites were epoxy or hydroxyl PUFAs, we developed a method to fully quantify specifically these families (supplemental Figs. S1, S2). We confirmed the increase in the concentration of 14-HDoHE, 17-HDoHE, and 8-HETE and the decrease of 9-HODE and 8(9)-EpEtrE in metacyclic compared with procyclic promastigotes (**Table 2**, supplemental Figs. S1, S2). In addition, we observed an increase in the concentration of 9,10-dihydroxyoctadecenoic acid, 9,10-DiHODE, 12-HODE, 11-HETE, 4-HDoHE, 20-HDoHE, and 19(20)-epoxydocosapentaenoic acid (EpDPE) (Table 2, supplemental Figs. S1, S2). The concentration of their precursors was not different between the two stages of the parasite (Fig. 1B), suggesting that the increase in metabolite concentration is not dependent on the amounts of precursors.

CYP-like proteins are responsible for PUFA metabolite production in *Leishmania*

In an attempt to elucidate whether the PUFA metabolism in *L. infantum* results from nonenzymatic autoxidation or from an enzymatic activity, metacyclic promastigotes were treated with different enzyme inhibitors. The treatment of metacyclic promastigotes with either NDGA, 17-ODYA, mammalian CYP ω-hydroxylase inhibitor (20), or clotrimazole and tioconazole, fungal CYP-dependent 14α-demethylase inhibitors (21, 22), impaired the production of most of the PUFA metabolites (**Fig. 2**). NDGA was the only inhibitor that decreased the concentration of all of the metabolites tested (Fig. 2). Other inhibitors decreased differentially the concentration of the different metabolites (Fig. 2). We highlighted five different profiles: *1*) 9-HODE, 13-HODE, and 14-HDoHE decreased by NDGA, 17-ODYA, and clotrimazole; *2*) 5-HETE, 12-HETE, and 5-oxo-ETE decreased only by NDGA; *3*) 8-HETE and 17-HDoHE decreased by NDGA and 17-ODYA; *4*) 15-HETE decreased by all of the inhibitors; and *5*) 8(9)-EpETrE decreased by NDGA, clotrimazole, and tioconazole.

The *Leishmania* genome database (GeneDB; Wellcome Trust Sanger Institute, Hinxton, UK; http://www.genedb.org) annotates only three putative CYP-like proteins coded by *LinJ.27.0090*, *LinJ.30.3610*, and *LinJ.34.3110* genes.

TABLE 2. PUFA metabolite concentration (nmol/1 × 10^8 parasites) in *L. infantum* at the procyclic or metacyclic promastigote stage

Metabolites	Procyclic	Metacyclic	*P*
9,10-DiHOME	17.68 ± 0.48	21.21 ± 0.69	0.0048
9,10-DiHODE	10.92 ± 0.21	12.01 ± 0.32	0.0298
15,16-DiHODE	5.83 ± 0.16	5.957 ± 0.12	0.8092
9-HODE	396.80 ± 34.93	249.91 ± 34.33	0.0298
10-HODE	14.24 ± 0.92	15.08 ± 0.75	0.4649
12-HODE	17.44 ± 0.81	19.32 ± 0.27	0.0298
13-HODE	962.11 ± 66.13	1,065.00 ± 67.89	0.3743
15-HODE	8.11 ± 0.10	7.41 ± 0.41	0.2198
5-HETE	26.29 ± 5.73	26.69 ± 4.25	1.0000
8-HETE	5.26 ± 0.29	13.68 ± 3.60	0.0048
11-HETE	3.87 ± 0.38	9.70 ± 0.90	0.0048
12-HETE	12.61 ± 3.94	12.15 ± 3.27	1.0000
15-HETE	43.57 ± 7.46	50.98 ± 1.15	0.7163
18-HETE	110.30 ± 7.48	112.01 ± 3.34	0.8092
5(*S*)-HETrE	0.970 ± 0.09	1.347 ± 0.13	0.1262
8(*S*)-HETrE	38.12 ± 6.69	44.35 ± 8.47	0.6879
12(*S*)-HETrE	12.25 ± 0.98	14.17 ± 0.93	0.1474
15(*S*)-HETrE	31.26 ± 2.36	39.37 ± 1.83	0.0766
8(9)-EpETrE	17.20 ± 2.07	2.91 ± 2.91	0.0247
4-HDoHE	146.10 ± 2.56	218.80 ± 17.02	0.0048
7-HDoHE	32.44 ± 2.44	37.94 ± 1.83	0.1262
8-HDoHE	114.80 ± 16.21	123.70 ± 10.91	0.1269
10-HDoHE	48.33 ± 3.24	47.18 ± 12.96	0.3760
11-HDoHE	63.44 ± 11.77	90.39 ± 8.89	0.1262
13-HDoHE	64.82 ± 3.81	64.05 ± 3.99	1.0000
14-HDoHE	112.10 ± 6.27	177.00 ± 18.81	0.0126
16-HDoHE	59.07 ± 3.22	69.35 ± 3.96	0.1262
17-HDoHE	129.1 ± 13.84	268.5 ± 39.05	0.0022
20-HDoHE	248.91 ± 24.76	327.50 ± 22.75	0.0431
7(8)-EpDPE	38.82 ± 3.271	46.52 ± 2.80	0.0646
10(11)-EpDPE	18.27 ± 1.89	24.83 ± 3.67	0.2403
13(14)-EpDPE	20.62 ± 2.43	19.75 ± 2.29	0.9360
16(17)-EpDPE	15.30 ± 2.08	19.19 ± 1.67	0.0646
19(20)-EpDPE	56.28 ± 5.09	69.91 ± 1.36	0.0301
10,11-DiHDPE	3.31 ± 0.29	3.64 ± 0.23	0.2598
13,14-DiHDPE	3.56 ± 0.30	3.99 ± 0.20	0.4616
16,17-DiHDPE	1.98 ± 0.63	2.22 ± 0.73	0.9348

DiHOME, dihydroxyoctadecenoic acid; HETrE, dihydroxyeicosatrienoic acid.

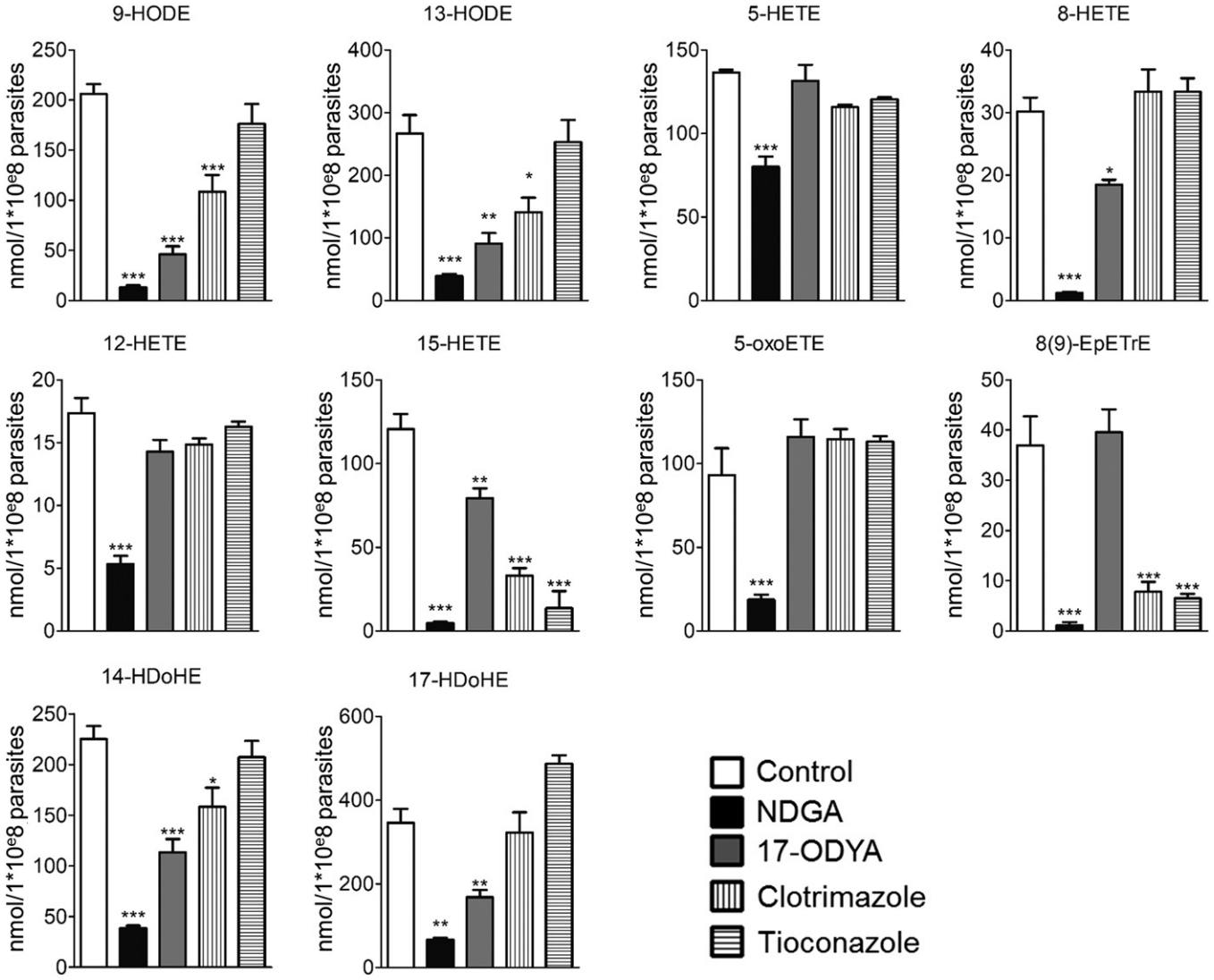
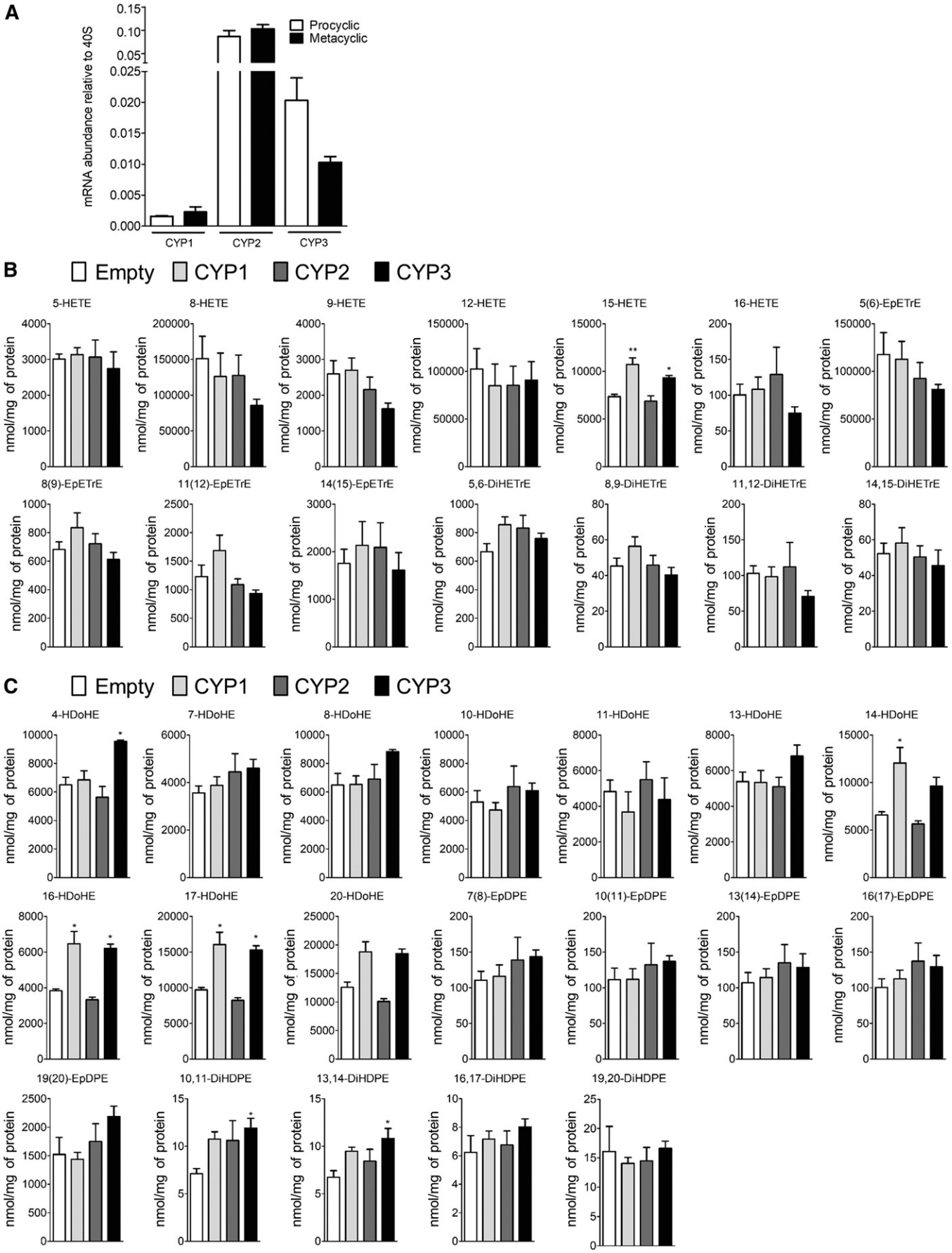


Fig. 2. Production of PUFA metabolites by metacyclic promastigotes is repressed by inhibitors of lipid metabolism. Production of PUFA metabolites by metacyclic promastigotes was assessed by LC/MS/MS after 24 h of treatment with 100 μM NDGA, 100 μM 17-ODYA, 10 μM clotrimazole, or 10 μM tioconazole compared with untreated metacyclic promastigotes. Data shown are from three experiments conducted in triplicate; means ± SDs are shown ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ significantly different from control group.

These genes, conserved among species, are described here as CYP1, CYP2, and CYP3, respectively. Our data confirm the expression of these three CYP enzymes in *Leishmania* at the two different stages (**Fig. 3A**). In order to further decipher the putative role of CYP1, CYP2, and CYP3 enzymes in the biosynthesis of PUFA metabolites, CHO cells expressing *Leishmania* recombinant CYP1, CYP2, or CYP3 enzymes were treated with either AA or DHA as fatty acid metabolite precursors, and the quantification of epoxy and hydroxyl PUFA metabolites was performed (Fig. 3B, C). Only 15-HETE (Fig. 3B) and 4-, 14-, 16-, and 17-HDoHE (Fig. 3C) were significantly increased in CYP1- and CYP3-transfected cells compared with untransfected cells after AA (Fig. 3B) or DHA treatment (Fig. 3C). In addition, transfection by CYP3 increased the concentration of 10,11- and 13,14-dihydroxyeicosatetraenoic acid (DiHDPE) (Fig. 3C). By contrast, the transfection of CHO cells with CYP2 had no effect on PUFA metabolite synthesis (Fig. 3B, C). Thus, CYP1 and CYP3 are implicated in the biosynthesis of AA- and DHA-derived metabolites in *Leishmania*.

Lipids extracted from *Leishmania* promote the synthesis of bioactive lipids in macrophages

To further explore the role of PUFA metabolites in the parasite/host interaction, lipids extracted from procyclic and metacyclic promastigotes were assessed for their ability to induce the synthesis of lipid mediators in mouse bone marrow-derived macrophages (BMDMs). PUFA metabolites quantified in the parasite alone [9- and 13-HODE; 5-, 8-, 12-, and 15-HETE; 8(9)-EpETrE; and 14- and 17-HDoHE] were increased in treated macrophages (**Fig. 4**). Treatment with the parasite lipid extracts did not modify the production of PGs such as PGD_2, 15d-PGJ_2, PGE_2, $PGF_{2\alpha}$, PGE_3, and 8-iso-PGA_2 or thromboxane B2, lipoxin A4, and 14(15)-EpETrE in BMDMs (supplemental Fig. S2). In contrast, the synthesis of the specialized proresolving mediators (SPMs)


A
mRNA abundance relative to 40S
Procyclic
Metacyclic
CYP1
CYP2
CYP3
B
Empty
CYP1
CYP2
CYP3
5-HETE
8-HETE
9-HETE
12-HETE
15-HETE
16-HETE
5(6)-EpETrE
8(9)-EpETrE
11(12)-EpETrE
14(15)-EpETrE
5,6-DiHETrE
8,9-DiHETrE
11,12-DiHETrE
14,15-DiHETrE
C
4-HDoHE
7-HDoHE
8-HDoHE
10-HDoHE
11-HDoHE
13-HDoHE
14-HDoHE
16-HDoHE
17-HDoHE
20-HDoHE
7(8)-EpDPE
10(11)-EpDPE
13(14)-EpDPE
16(17)-EpDPE
19(20)-EpDPE
10,11-DiHDPE
13,14-DiHDPE
16,17-DiHDPE
19,20-DiHDPE
nmol/mg of protein

RvD2, PDx, and 7-MaR1 was significantly higher in BMDMs treated with metacyclic lipid extracts than those treated with procyclic lipid extracts (Fig. 4B).

Lipids extracted from the metacyclic stage of *Leishmania* promote the polarization of macrophages into a proresolving M2 phenotype

M0 macrophages derived from bone marrow were treated with lipids extracted from the two stages of the parasite. Only metacyclic lipid extracts induced the transcriptional upregulation of the arginase 1 (*Arg1*) and *CD206* genes, two bona fide markers of the alternatively activated M2 macrophage phenotype (**Fig. 5A**) (23). At the same time, no change in the expression level of the main markers of M1 macrophage phenotypes was observed, irrespective of parasite stages. When M0 macrophages were treated with lipids extracted from the two stages of the parasite concomitantly with INF-γ to induce M1 differentiation, only metacyclic lipid extracts decreased several M1 markers induced by IFN-γ treatment (Fig. 5B).

DISCUSSION

Metacyclogenesis is known to be crucial for infectivity acquisition by promastigotes. Indeed, metacyclic promastigotes are primarily characterized by their ability to infect macrophages (6, 7, 24, 25) thanks to a higher resistance to host complement-mediated lysis, which is necessary to save time to infect macrophages. This is essentially due to the structure and size of the surface lipophosphoglycan of metacyclic promastigotes (25, 26) and the inactivation of C3b by leishmanolysin (gp63), a protein that is not present in procyclic promastigotes (4). Our study describes the ability of metacyclic promastigotes to promote their own survival by modifying the host macrophage phenotype through the synthesis of specific PUFA metabolites.

We first described the production by *L. infantum* of 10 different PUFA metabolites derived from LA (18:2n-6), AA (20:4n-6), and DHA (22:6n-3). The relative abundance of these different metabolites is in agreement with the abundance of their precursors (from least to most abundant: AA, dihomo-γ-linolenic acid, DHA, α-linolenic acid, and LA) (27). Contrary to its mammalian host, *Leishmania* is able to produce LA from oleic acid (18:1n-9) via an oleate desaturase. LA is then desaturated either to γ-linolenic acid (18:3n-6) by Δ6 desaturase activity or to α-linolenic acid (18:3n-3) by the linoleate (Δ15) desaturase. The α-linolenic acid is the major accumulated product in *Leishmania* (28, 29), which explains the unique DHA-AA ratio quantified in *Leishmania* as DHA derived from the α-isomer and AA from the γ-isomer. Independent of the amount of AA and DHA, which is similar in metacyclic promastigotes compared with procyclic promastigotes, our results highlight the overproduction of different PUFA metabolites in metacyclic promastigotes. The use of NDGA, mainly known to inhibit mammalian 5-lipoxygenase, highly repressed the synthesis of all the PUFA metabolites in *L. infantum*. Due to the absence of the lipoxygenase coding gene in the *Leishmania* genome, we further assessed whether the NDGA inhibitory effect on PUFA metabolite synthesis was dependent on its known activity as a nonspecific oxidase/reductase inhibitor or CYP inhibitor (30). Treatment with different CYP inhibitors differentially decreased the different PUFA metabolites. In mammalian cells, CYP ω-hydroxylases provide pathways for the production of bioactive metabolites from AA such as 18-, 19-, and 20-HETE mainly by the CYP4F family (31–33). Interestingly, 17-ODYA, which decreased the concentration of several metabolites in *Leishmania*, is a suicide inhibitor of CYP4F, indicating possible ω-hydroxylase activity present in the parasite (32). In addition, CYP2C8 and CYP2C9, characterized by their epoxygenase activity, may also produce HETE and HODE by bisallylic hydroxylation activity (34). These results, in line with previous studies, suggest that CYP enzymes may participate in the synthesis of PUFA metabolites in *Leishmania*. Even though there are no studies available addressing the functional involvement of CYP in PUFA metabolism in *Leishmania*, CYP enzymatic activities were described in trypanosomatids, including *Leishmania sp.* (35). A pivotal role of this enzyme in *Leishmania* biology is supported by the antileishmanial activity of the azole antifungals, which are known to act as CYP inhibitors (36–38). The *Leishmania* genome database (GeneDB, Wellcome Trust Sanger Institute, Hinxton, UK) annotates only three putative CYP-like proteins, described here as CYP1, CYP2, and CYP3. This group of proteins is annotated as membrane-associated proteins with monooxygenase activity; however, there are limited experimental data focusing on their mechanisms of action and function in the *Leishmania* life cycle. CYP1, classified as CYP5122A1 by the Cytochrome P450 Nomenclature Committee, has been reported to play a key role in ergosterol metabolism and to be associated with mechanisms of drug resistance in *L. donovani* (39) as well as in cell growth and infectivity (40). Promastigotes heterozygote for *CYP1* gene deficiency show a lesser capacity to differentiate into metacyclic forms and to subsequently cause infection (40). CYP2 seems to be underregulated in *L. infantum* axenic amastigotes during amastigote morphogenesis (41). By expressing *Leishmania* recombinant CYP1, CYP2, or CYP3 enzymes in CHO cells, we demonstrated the ability of CYP1 and CYP3 to synthetize PUFA metabolites, mainly hydroxylated DHA and in particular 4-, 14-, 16-, and 17-HDoHE. The concentration

Fig. 3. CYP-like proteins are responsible for PUFA metabolite production in *Leishmania*. A: mRNA expression of *CYP1*, *CYP2*, and *CYP3* genes in procyclic and metacyclic promastigotes determined by RT-qPCR. Data shown are from five experiments conducted in triplicate; means ± SEMs are shown ($n = 5$). B: Concentration of AA-derived metabolites in CHO cells expressing recombinant CYP1, CYP2, or CYP3 determined after cell media supplementation with AA. Data shown are from six experiments conducted in duplicate; means ± SEMs are shown ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from the empty group. C: Concentration of DHA-derived metabolites in CHO cells expressing recombinant CYP1, CYP2, or CYP3 determined after cell media supplementation with DHA. Data shown are from six experiments conducted in duplicate; means ± SEMs are shown ($n = 6$). $^{*}P < 0.05$ significantly different from the empty group.

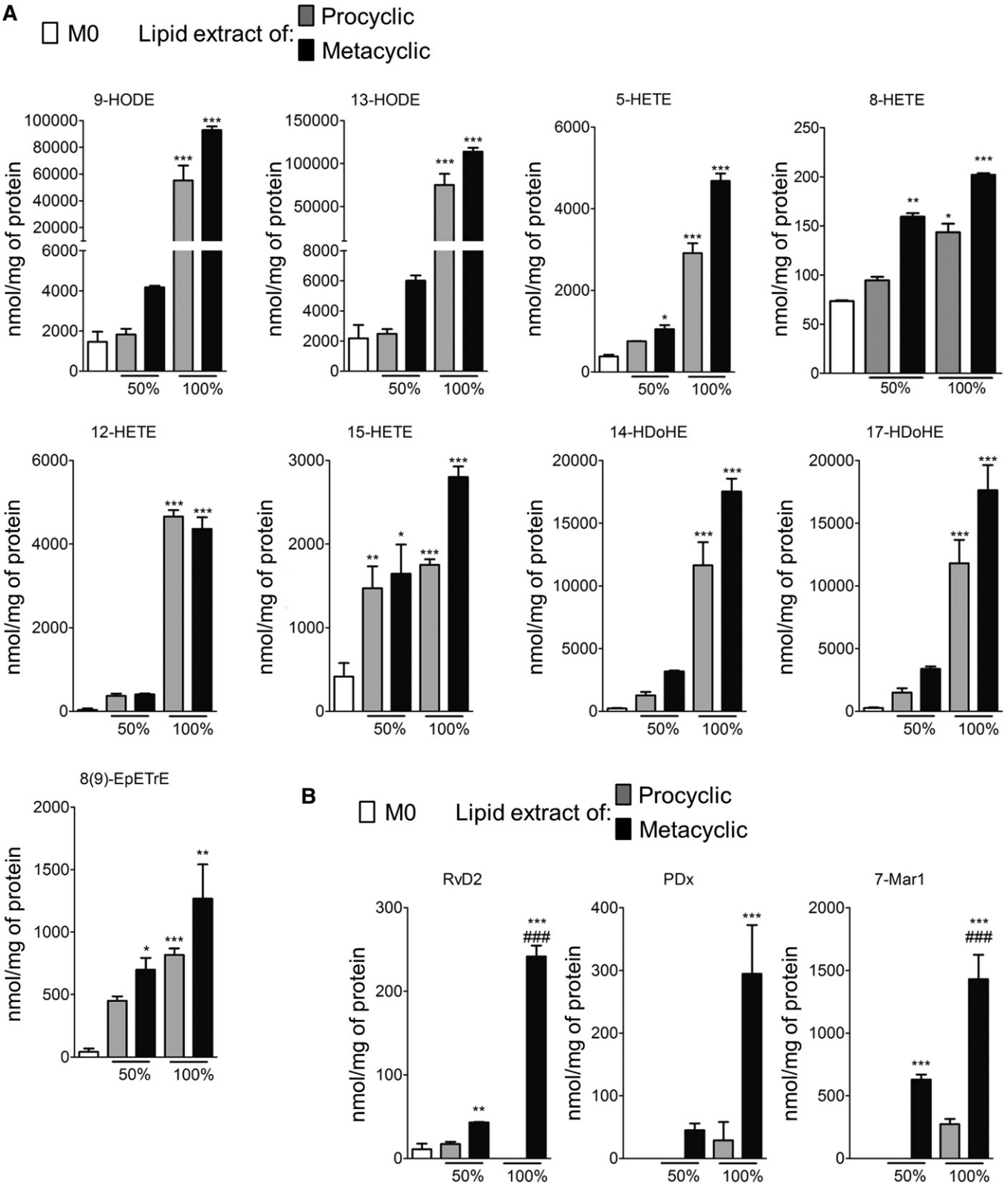


Fig. 4. Lipids extracted from *Leishmania* promote the synthesis of lipid mediators by BMDMs. A: Production of PUFA metabolites assessed by LC/MS/MS in BMDMs treated with 0% (M0), 50%, or 100% of lipids extracted from 1×10^8 procyclic and metacyclic promastigotes. Data shown correspond to three experiments conducted in triplicate; means ± SEMs are shown ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ significantly different from the M0 group. B: Production of proresolving mediators assessed by LC/MS/MS in BMDMs treated with 0% (M0), 50%, or 100% of lipids extracted from 1×10^8 procyclic and metacyclic promastigotes. Data shown are from three experiments conducted in triplicate; means ± SEMs are shown ($n = 3$). $^{**}P < 0.05$ and $^{***}P < 0.01$ significantly different from the M0 group and $^{\#\#\#}P < 0.001$ significantly different from the lipid extracts of the procyclic group.

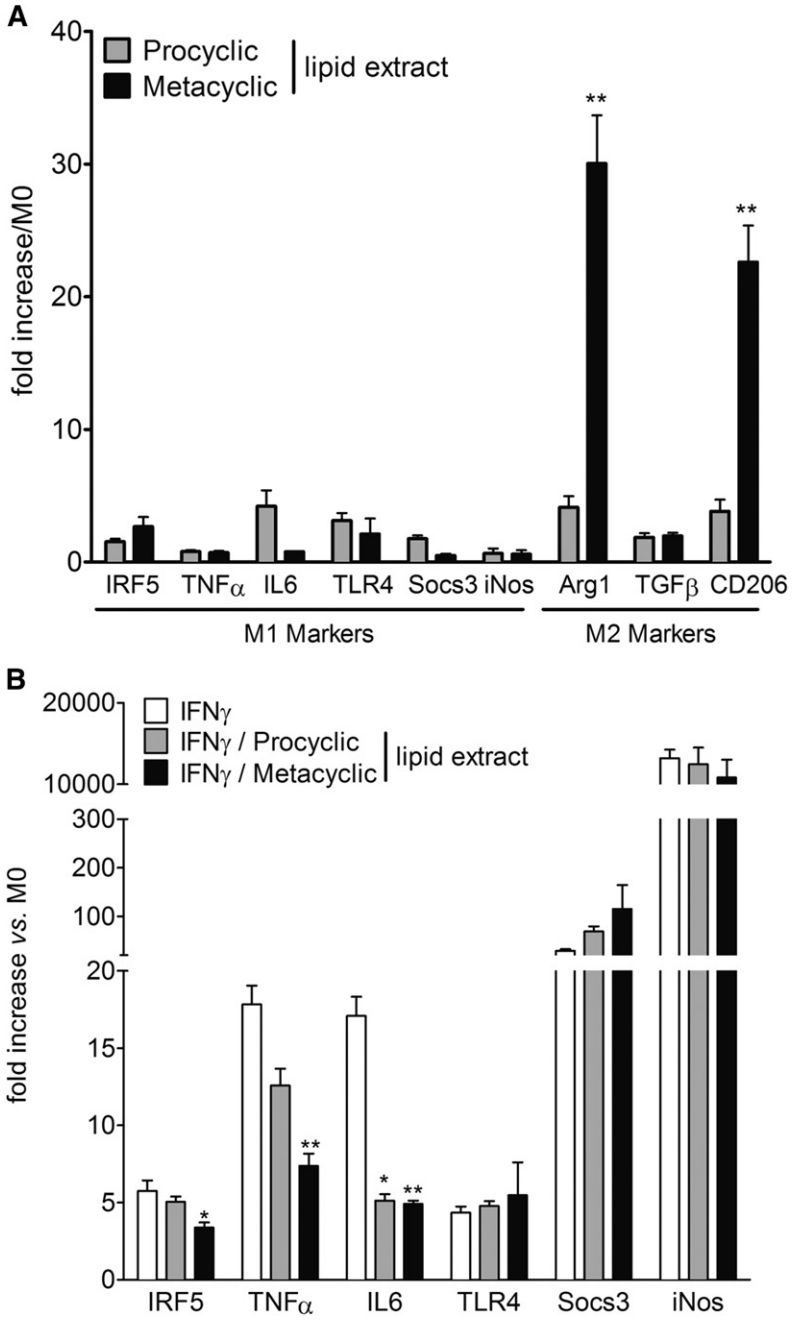


Fig. 5. Lipids extracted from metacyclic promastigotes promote the polarization of macrophages into a proresolving M2 phenotype. A: mRNA expression of markers of M1 and M2 polarization quantified in BMDMs after treatment with lipid metabolites from procyclic and metacyclic promastigotes. Data shown are means of fold changes ± SEMs compared with the undifferentiated monocyte (M0) from three independent experiments conducted in triplicate ($n = 3$). $^{**}P < 0.01$ significantly different from the procyclic lipid extract group. B: Gene expression of M1 and M2 markers in BMDMs after treatment with lipid extracts from procyclic and metacyclic promastigotes and/or IFN-γ. Data shown are means of fold changes ± SEMs compared with the undifferentiated monocyte (M0) from three independent experiments conducted in triplicate ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from the IFN-γ group. iNOS, inducible nitric oxide synthase; IRF5, IFN regulatory factor 5; Socs3, suppressor of cytokine signaling 3.

of several PUFA metabolites, increased in metacyclic promastigotes, was not affected by the transfection of the different CYPs in CHO cells, demonstrating that other enzymes may be implicated in PUFA metabolite synthesis. Among them, it is important to note the increase in 19(20)-EpDPE concentration, which plays a critical role in monocyte lineage recruitment during inflammatory resolution (42).

We also demonstrated that lipids extracted from metacyclic promastigotes did not modify the production of PGs by macrophages but were able to increase the synthesis of the SPMs RvD2, PDx, and 7-MaR1. This increase in SPMs could result from the increase of their precursors (supplemental Fig. S2), 14-HDoHE for maresin and 17-HDoHE for resolvin and protectin (43). Our results are in agreement with a previous study demonstrating a key role of RvD1 in diffuse cutaneous leishmaniasis and in intracellular *L. amazonensis* replication in human macrophages (44). Upon parasitic challenge, it has been shown that *Leishmania* actively manipulated the metabolic pathway to avoid the M1 polarization of macrophages (7). We assessed the involvement of PUFA metabolites from the parasite in this process. Lipid extracts from metacyclic promastigotes were also able to induce transcriptional upregulation of markers of the alternatively activated M2 macrophage phenotype (Fig. 5A). Our results are in agreement with the mouse model of adipose tissue inflammation, in which DHA and RvD1 intake induces a shift of the macrophage phenotype with an increase in *Arg1* and *CD206*, concomitantly with a downregulation of *TNF-α* and *IL-6* expression (45). Transcriptional levels of *TNF-α*, *IL-6*, and the typical transcriptional factor of the M1 phenotype induced by IFN-γ, IFN regulatory

factor 5, decreased after coincubation with parasitic lipid extracts from the infective form of *Leishmania*. In *L. major*-infected mice, TNF-α inhibition is associated with the upregulation of *Arg1* and *CD206* without affecting inducible nitric oxide synthase expression (26). The balance in favor of Arg1 rather than inducible nitric oxide synthase represses reactive oxygen species production (46) and increases polyamine biosynthesis, which is essential for the intracellular growth of *Leishmania* (26, 47). Our data demonstrate that DHA- and AA-derived metabolites from metacyclic promastigotes also induce a minimal proinflammatory response in the host to favor parasite growth. Therefore, metabolites derived from DHA and specifically overrepresented in metacyclic promastigotes (14- and 17-HDoHE) could participate in building a safer environment to facilitate infection by affecting the polarization of immune response during infection.

The results presented here demonstrate the production of PUFA-derived metabolites in the *Leishmania* parasite. Biosynthesis of these metabolites partially depends on *Leishmania* CYP-like protein (CYP1 and CYP3) activity. Infectivity acquisition is accompanied by an increased production of 14- and 17-HDoHE, precursors of the proresolving lipid mediators maresin, D-series resolvin, and protectin (43). The treatment of macrophages with lipids extracted from the infective forms polarizes macrophages into an M2 phenotype. M2 macrophages produce high levels of proresolving bioactive lipids (RvD2, 7-Mar1, and PDx) and several markers of proresolving phenotypes as CD206 or Arg1, promoting the survival and proliferation of *Leishmania* inside the host cell.

The authors thank Jean-Luc Parrou for his advice on CYP cloning. The authors also thank Genotoul, Anexplo, US006/INSERM, the University of Toulouse, and the INSERM MetaToulLipidomics Facility-MetaboHUB ANR-11-INBS-010, where lipidomic analysis was performed.

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