RESEARCH ARTICLE

Crude leaf extracts of Piperaceae species downmodulate inflammatory responses by human monocytes

Angela Carolina Finato¹, Thais Fernanda Fraga-Silva¹,², Amanda Uliana Carvalho Prati³, Amauri Alves de Souza Júnior⁴, Bruna Fonseca Mazzeu³, Lidiane Gaspareto Felippe³, Rute Alves Pinto³, Marjorie de Assis Golim⁴, Maria Sueli Parreira Arruda¹, Maysa Furlan³, James Venturini¹,⁵*

¹ Universidade Estadual Paulista (Unesp), Faculdade de Ciências, Bauru, SP, Brazil, ² Universidade Estadual Paulista (Unesp), Instituto de Biociências, Botucatu, SP, Brazil, ³ Universidade Estadual Paulista (Unesp), Instituto de Química, Araraquara, SP, Brazil, ⁴ Universidade Estadual Paulista (Unesp), Faculdade de Medicina, Botucatu, SP, Brazil, ⁵ Universidade Federal de Mato Grosso do Sul (UFMS), Faculdade de Medicina, Campo Grande, MS, Brazil

* james.venturini@ufms.br

Abstract

In this study, we aimed to evaluate the immunomodulatory effects of crude leaf extracts from Piper gaudichaudianum Kunth, P. arboresum Aub., P. umbellata L., P. fulgineum Kunth, and Peperomia obtusifolia A. Dietr. on an in vitro model of inflammatory response. The crude extracts were previously obtained by maceration of the leaves. The half-maximal inhibitory concentration was determined by the MTT assay using human peripheral blood mononuclear cells. Human monocytes were simultaneously challenged with each crude extract and lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, to induce a strong inflammatory response. After 24 h of incubation, cell-free supernatants were used for evaluating the mediators involved in inflammation: H₂O₂, TNF-α, IL-8, IL-6, IL-1β, IL-10, IL-12, FGF-b, and TGF-β1. We also compared the results with the effects of ketoprofen, a well-known anti-inflammatory drug. The P. gaudichaudianum crude extract downmodulated the production of H₂O₂, IL-1β, IL-6, IL-8, and TGF-β1 by LPS-stimulated monocytes; P. arboresum, IL-1β, IL-6, IL-8, and TNF-α; P. umbellata and P. fulgineum, H₂O₂, IL-1β, IL-6, IL-8, IL-10, and TNF-α; and P. obtusifolia, H₂O₂, IL-6, IL-8, IL-10, and TNF-α. In general, the crude leaf extracts amplified the anti-inflammatory response when compared with ketoprofen, particularly reducing the production of IL-8, a mediator involved in neutrophil recruitment during tissue damage. Thus, the crude leaf extracts of P. gaudichaudianum, P. arboresum, P. umbellata, P. fulgineum, and Peperomia obtusifolia elicited an anti-inflammatory response against LPS-challenged monocytes. These findings show the anti-inflammatory properties of these crude leaf extracts and offer new perspectives for their use in the treatment of inflammatory diseases.
Introduction

The family Piperaceae comprises pantropical herbal plants, widely distributed in Latin America, particularly from Mexico to the southwest of Argentina [1–3]. The family is composed of five genera: *Piper*, *Peperomia*, *Manekia*, *Zippelia*, and *Verhuellia* [4,5]. *Piper* and *Peperomia* are the most representative genera with 2000 and 1700 species, respectively [6,7]. Besides their economic importance, Piperaceae species have been used in traditional medicine as an anti-inflammatory agent, for relief from toothache, gynecological illnesses, and intestinal disorders, as well as psychotropic and anxiolytic agents [8]. *Piper* and *Peperomia* species possess various classes of bioactive compounds, such as amides [9,10], lignans [11,12], secolignans [13,14], phenylpropanoids [15], prenylated benzoic acid derivatives [16], chromenes and chromanes [17–21], terpenes [22], alkaloids [23–25], and others [26,27].

Previous chemical and biological studies on Piperaceae have revealed them to be a rich source of new biologically active secondary metabolites. The accumulation of a major secondary metabolite, 4-nerolidylcatechol, was observed in *P. umbellata* [syn. *Pothomorphe umbellata* (L.) Miq., *Heckeria umbellata* (L.) Kunth., *Piper hilarianum* Stend] leaves [28]. The metabolite exhibits potent anti-oxidant and anti-inflammatory activities [29]. The potent inhibitory effect of kavalactones in *P. fuligineum* against hepatitis C virus replication was recently described [30]. Their potential anti-inflammatory and anxiolytic properties were also described [31]. *P. arboreum* possesses antifungal, trypansomidal, antimicrobial, and anti-oxidant pyrrolidine amides as major natural compounds [9,32–34]. The accumulation of prenylated chromenes and chromanes with potent trypansomidal activity against the Y-strain of *Trypanosoma cruzi* was observed in *P. gaudichaudianum* and *Peperomia obtusifolia* [18,20,21]. Chemical studies with *P. gaudichaudianum* demonstrated the presence of gaudichaudianic acid, a prenylated chromene that is a major secondary metabolite in leaves and roots of this species [18,19]. In terms of biological activities, this compound showed potent trypansomidal and antifungal activities against plant pathogens. Furthermore, the unusual presence of two natural isomeric forms of gaudichaudianic acid [(+)-S and (-)-R] was observed during the isolation of such compounds, as well as their synergistic effect, with the racemic mixture being the most active in trypansomidal assays [20].

In this study, we revealed new biological properties of the crude leaf extracts of *P. gaudichaudianum* Kunth, *P. arboreum* Aub., *P. umbellata* L., *P. fuligineum* Kunth, and *Peperomia obtusifolia* A. Dietr., by characterizing their immunomodulatory effects on an in vitro model of human inflammatory response.

Material and methods

Plant material

*P. gaudichaudianum* leaves were collected from the campus of the University of São Paulo, Brazil, and identified by Dr. Inês Cordeiro (Botanic Garden curator of University of São Paulo, Brazil). A voucher specimen (Kato-0093) has been deposited at the Herbarium of the Botanic Institute, São Paulo, Brazil. *P. arboreum*, *P. umbellate*, and *Peperomia obtusifolia* leaves were collected from the greenhouse of the Institute of Chemistry of UNESP, Araraquara, SP, Brazil, and identified by Dr. Inês Cordeiro and Dr. G. E. D. Paredes (University of Pedro Ruiz Gallo, Peru), respectively. Voucher specimens [(Cordeiro-1936), (Kato-671), (Kato-070), respectively] have been deposited at the Herbarium of the Botanic Institute of University of São Paulo, Brazil. *P. fuligineum* leaves were collected from the Botanic Garden, Araraquara, São Paulo, Brazil, and identified by Dr. Inês Cordeiro. A voucher specimen (Kato-0720) has been deposited at the Herbarium of the Botanic Garden of the University of São Paulo, Brazil.
Preparation of crude extracts

The preparation of the ethanolic extracts and chemical characterizations of *P. gaudichaudianum*, *P. arboreum*, and *P. umbellata* have been previously described [29,35,36]. Briefly, leaves of these species were milled, extracted with ethanol (EtOH), and the extract concentrated under vacuum to yield the crude extracts.

Dried leaves of the *P. fuligineum* were milled and extracted with ethanol (EtOH). This ethanolic extract was concentrated under vacuum to obtain 54.4 g of the concentrate, which was resuspended in MeOH:H2O (4:1) and partitioned with hexane, CHCl3, and EtOAc successively. The soluble CHCl3 fraction (13 g) was subjected to bioassay. Dried leaves (430 g) of *Peperomia obtusifolia* were milled and extracted by maceration at room temperature with EtOAc (3 × 1000 mL) for 72 h. The resulting solution was filtered and concentrated under reduced pressure to obtain 21 g of crude extract. The EtOAc extract was subjected to bioassay. Final stock concentrations of extracts were 100 mg/mL.

Experimental design

The study was performed in two steps. First, we evaluated the cytotoxicity and inhibitory concentration of 50% (IC50) of each crude extract using peripheral blood mononuclear cells (PBMCs). Then, we evaluated the immunomodulatory properties of the five Piperaceae species using a widely known in vitro model for lipopolysaccharide (LPS)-mediated inflammatory response. LPS is an endotoxin of Gram-negative bacteria that triggers an intense release of pro-inflammatory cytokines by monocytes. For each assay, cells were stimulated with ketoprofen, an anti-inflammatory drug. Human monocytes were cultivated and subjected to six treatments: (1) medium (unstimulated control), (2) LPS, (3) crude extracts (*P. gaudichaudianum*, *P. arboreum*, *P. umbellata*, *P. fuligineum* and *Peperomia obtusifolia*), (4) LPS + crude extracts, (5) ketoprofen, and (6) LPS + ketoprofen. Each assay was performed in duplicate or triplicate.

Isolation of PBMCs

Human peripheral venous blood was obtained from six healthy donors. The blood was collected with Vacutainer® tubes containing heparin as an anticoagulant. PBMCs were isolated by density gradient centrifugation on Histopaque®-1077 (Sigma-Aldrich, St Louis, MO, USA). PBMCs were centrifuged and resuspended in 1.0 mL of RPMI-1640 (Nutricell, Campinas, SP, Brazil) supplemented with 20% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Grand Island, NY, USA), penicillin (100 U1 mL), and streptomycin (100 mg mL) (Gibco). Cell viability, as determined by 0.2% trypan blue, was >95% in all experiments. Concentration was adjusted to 1.0 × 10^6 cells/mL using Turk stain.

Cytotoxicity and IC50

Cytotoxic activity of the crude extracts was determined by the colorimetric microculture 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [37]. PBMCs (1.0 × 10^5 / well) were seeded into 96-well flat bottom plates, cultured in the presence of each crude extract dissolved in dimethyl sulfoxide (DMSO), and serially diluted in phosphate buffered solution (PBS) (0.156, 0.313, 0.625, 1.25, 2.5, 5.0 mg/mL). The final concentration of DMSO at each crude extract was always less than 1%. Concanavalin A (10 mg/mL), a potent mitogen for T lymphocytes, was used as the internal control for lymphoproliferation. After continuous incubation for 96 h at 37°C under 5% CO2 atmosphere, the culture plate was centrifuged for 5 min at 1500 rpm and supernatants were replaced with 20.0 μL of MTT solution (5.0 mg/mL) plus 100.0 μL of supplemented RPMI-1640 medium. After incubation for 2 h, the
supernatant was removed, and formazan crystals that formed in viable cells were dissolved in 100.0 μL of DMSO per well. The optical densities were measured using an ELISA microreader (EL800, BIO-TEK Instruments, INC) at a wavelength of 540 nm. The cytotoxic index was determined by the ratio between treated cells and non-treated cells. The IC<sub>50</sub> was determined by linear regression analysis.

**Monocyte cell culture**

Mononuclear cells were obtained as previously described. To obtain human monocytes, mononuclear cells were counted and adjusted to 1.0 × 10<sup>6</sup> of mononuclear phagocytes/mL. The viability was higher than 90%, as judged by the uptake of 0.02% neutral red (Sigma-Aldrich). The cells were distributed in a volume of 100 μL per well into 96-well flat bottom plates and incubated for 2 h at 37˚C in a humid atmosphere with 5% CO<sub>2</sub> to allow monocytes to adhere. Non-adherent cells were removed by washing the wells thrice with RPMI-1640 supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 mg/mL) and the remaining monocytes (>90% mononuclear phagocytes as assessed by morphological examination and expression of CD14, CD19 and CD3 by fluorescence-activated cell sorting) were used for experiments. In order to evaluate the influence of compounds in an inflammatory environment, we next subjected monocytes to six treatments, as following: (1) medium (unstimulated control), (2) LPS– 10 μg/mL, (3) each crude extracts at concentration of IC<sub>50</sub>, (4) LPS + crude extracts, (5) ketoprofen– 5.2 mM [38], and (6) LPS + ketoprofen. All the substances and compounds were added simultaneously. Culture cells were incubated at 37˚C under 5% CO<sub>2</sub> atmosphere for 24 h. At the end of the cell culture period, the supernatants were removed and stored at– 80˚C for determination of cytokines and the hydrogen peroxide release assay.

**Hydrogen peroxide release**

At the end of the cell culture period, the monocytes were incubated with phenol red solution [dextrose 1% (Sigma-Aldrich), phenol red 1% (Sigma-Aldrich), horseradish peroxidase type II– 5 U (Sigma-Aldrich)] and plated at 37˚C under 5% CO<sub>2</sub> atmosphere for 1 h according to the method described by Russo <i>et al</i> [39]. The reaction was stopped by the addition of 1.0 N NaOH and the H<sub>2</sub>O<sub>2</sub> concentration was determined using an ELISA microreader at 620 nm.

**Cytokine analysis**

The levels of interleukin (IL)-8, IL-1β, IL-6, IL-10, TNF-α, and IL-12 p70 were determined by flow cytometry using a BD cytometric bead array (CBA) (BD Biosciences, CA, USA). The levels of transforming growth factor beta (TGF-β) and basic fibroblast growth factor (basic FGF) were determined by ELISA using a cytokine Duo-Set Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

**Statistical analysis**

All experimental protocols were performed at least three times. The results of the controls (medium and ketoprofin, unstimulated and LPS stimulated) were used in the analyses for all extract evaluated. Comparison of the treatments was performed by repeated measures ANOVA with Bonferroni post-test. The analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and statistical significance was set at p-value < 0.05.
Ethics Committee

This study was approved by the Research Ethics Committee of the Faculdade de Ciências de Bauru—UNESP (Certificate of Presentation for Ethical Consideration—CAAE #20497414.0.0000.5398). Written informed consent to participate and publish the data was obtained and signed by all the participants.

Results

Table 1 presents the IC$_{50}$ for each crude extract. The values ranged from 0.24 mg/mL to 2.19 mg/mL. The complete results of the cytotoxicity assay are shown in S1 Fig.

The effects of the crude leaf extracts were evaluated using an *in vitro* inflammatory milieu triggered by LPS. As expected, the LPS-stimulated monocytes exhibited higher production of hydrogen peroxide ($\text{H}_2\text{O}_2$) ($p = 0.01$), IL-1β ($p = 0.01$), TNF-α ($p = 0.016$), IL-10 ($p = 0.04$), and TGF-β1 ($p = 0.04$) than non-stimulated monocytes (Figs 1–5). These findings support our *in vitro* system in determining immunomodulatory properties.

To evaluate the effects of crude leaf extract on an inflammatory milieu, we first evaluated the influence of each crude extract on human monocytes without an inflammatory stimulus, i.e., we verified if the crude extracts triggered any alteration in monocyte activity at baseline condition, and we also compared the effects of the crude extracts with those of ketoprofen. Next, using the inflammatory *in vitro* model, we compared the effects of crude leaf extracts to those of placebo treatment. Then, we compared them to those of ketoprofen treatment to identify the efficacy of crude extract over anti-inflammatory drugs.

Compared to that in unstimulated monocytes, the crude extracts of *P. gaudichaudianum* abrogated the production of $\text{H}_2\text{O}_2$, IL-6, TNF-α, IL-8, and IL-10 (baseline) (Fig 1). Lower production of $\text{H}_2\text{O}_2$, IL-1β, IL-6, IL-8, and TGF-β1 was observed in LPS-stimulated monocyte cell culture treated with crude extract compared to that in placebo treatment (Fig 1). The anti-inflammatory action of the crude extract was higher than ketoprofen in relation to the production of $\text{H}_2\text{O}_2$, IL-6, and IL-8 by LPS-challenged monocytes (Fig 1).

Compared to that in unstimulated monocytes, the crude extract of *P. arboreum* abrogated the production of IL-8 (Fig 2). Lower production of IL-1β, IL-6, IL-8, and TNF-α was observed in LPS-stimulated monocyte cell culture treated with the extracts than that observed in the placebo treatment (Fig 2). The anti-inflammatory action of the crude extract was higher than ketoprofen in relation to the production of $\text{H}_2\text{O}_2$, IL-6, IL-8, and TGF-β1 by LPS-challenged monocytes (Fig 2).

Compared to that in unstimulated monocytes, the crude extract of *P. umbellata* abrogated the production of $\text{H}_2\text{O}_2$, IL-1β, IL-6, TNF-α, IL-8, and IL-10 (Fig 3). Lower production of $\text{H}_2\text{O}_2$, IL-1β, IL-6, IL-8, IL-10, and TNF-α was observed in LPS-stimulated monocytes treated with the extracts than in the placebo treatment (Fig 3).

The anti-inflammatory action of the crude extract was higher than ketoprofen in relation to the production of IL-6, IL-8, and TNF-α by LPS-challenged monocytes (Fig 3).

Table 1. IC$_{50}$ values of the crude extracts of *P. gaudichaudianum*, *P. arboreum*, *P. umbellata*, *P. fuligineum* and *Peperomia obtusifolia*.

| Crude extracts          | IC$_{50}$ (mg/mL) |
|-------------------------|-------------------|
| *P. gaudichaudianum*    | 0.55              |
| *P. arboreum*           | 2.19              |
| *P. umbellata*          | 1.56              |
| *P. fuligineum*         | 0.24              |
| *Peperomia obtusifolia* | 2.12              |

https://doi.org/10.1371/journal.pone.0198682.t001
Compared to that in unstimulated monocytes, the crude extract of *P. fuligineum* abrogated the production of H$_2$O$_2$, TNF-α, IL-8, and IL-10 (Fig 4). Lower production of H$_2$O$_2$, IL-1β, IL-6, IL-8, IL-10, and TNF-α was observed in LPS-stimulated monocytes treated with the extracts than in the placebo treatment (Fig 4). The anti-inflammatory action of the crude extract was higher than ketoprofen in relation to the production of IL-6, IL-8, TGF-β1, and TNF-α by LPS-challenged monocytes (Fig 4).

Compared to that in unstimulated monocytes, the crude extract of *Peperomia obtusifolia* abrogated the production of H$_2$O$_2$, IL-6, TNF-α, IL-8, and IL-10 (Fig 5). Lower production of H$_2$O$_2$, IL-6, IL-8, IL-10, TNF-α, and a tendency toward lower production of IL-1β was observed in LPS-stimulated monocytes treated with the extracts than in the placebo treatment (Fig 5). The anti-inflammatory action of the crude extract was higher than ketoprofen in relation to the production of IL-6, IL-8 TGF-β1, and TNF-α by LPS-challenged monocytes (Fig 5).

In all experimental protocols, the levels of IL-12p40 and FGF-b were below the detection limit.

**Discussion**

To the best of our knowledge, we demonstrated for the first time, in this study, that all of the evaluated crude leaf extracts induced anti-inflammatory activity that was more potent than that of ketoprofen. Compared with ketoprofen, the evaluated crude extracts significantly reduced the production of crucial pro-inflammatory markers, such as IL-6, IL-8, and TNF-α. IL-8 or CXCL8, also known as neutrophil chemotactic factor, is a chemokine involved in neutrophil recruitment and degranulation during tissue injury response [40]. During inflammation, IL-6 induces T and B cell and macrophage differentiation, acute phase proteins synthesis, and T cell activation [40]. TNF-α is an important pro-inflammatory cytokine that can induce...
In the present study, we observed that LPS-stimulated monocytes treated with crude extracts of *P. gaudichaudianum* and *P. fuligineum* also downregulated the production of TGF-β1 compared to LPS-stimulated untreated monocytes. TGF-β1 is a pleiotropic growth factor usually associated with anti-inflammatory and regulatory properties; however, it presents a crucial role in fibrotic diseases, including IPF [45], sarcoidosis [46], and hepatic fibrosis [47]. Infiltrating monocytes act as important sources of TGF-β1 during early fibrogenesis [48]. Thus, *P. gaudichaudianum* and *P. fuligineum* seem to present an interesting antifibrotic potential.

Reduced production of IL-1β was observed in LPS-stimulated monocytes treated with each crude extract compared to that in monocytes that were subjected to the inflammatory milieu but did not receive any treatment. This condition reinforced the anti-inflammatory potential of *P. gaudichaudianum*, *P. arboreum*, *P. umbellata*, *P. fuligineum*, and *Peperomia obtusifolia*, since IL-1β is one of the most important pro-inflammatory cytokines involved in many inflammatory/infection diseases [49,50].

Another important finding was the capacity of all extracts to prevent the production of IL-10 by monocytes, as well as by monocytes treated with ketoprofen. Usually, the production of IL-10 is increased by LPS-stimulated monocytes [51,52], probably due to an effort of cells to control the inflammation. Indeed, sepsis is associated with a monocyte hyporesponsiveness to LPS that appears to be proportional to the severity of sepsis and release of cytokines such as...
PGE2, TGF-β, and IL-10 [53]. Therefore, the decreased levels of IL-10 induced by the crude extracts in comparison to non-treated LPS-stimulated monocytes reinforce the idea that the compounds could better control the inflammation in our \textit{in vitro} system.

Few studies have addressed the immune-related effects of the Piperaceae species. The ethanolic extract of the leaves of \textit{Piper betle} Linn, which showed the accumulation of allylpyrocatechol glycosides, chavibetol glycosides, allylpyrocatechol, and chavibetol as the major chemical constituents, triggers \textit{in vitro} downregulation of transcription of inducible nitric oxide synthase and low production of IL-12 by rat peritoneal phagocytes [48]. These studies also confirmed the immunomodulator y effect of the extract in the complete Freund’s adjuvant-induced model of arthritis in rats [54]. The aqueous extract of the aerial portion of \textit{Peperomia pellucida} (L.) HBK had an anti-inflammatory effect in the \textit{in vivo} model of paw edema, induced by carrageenan, thereby interfering with prostaglandin synthesis [55]. Although the studies did not characterize the extract chemically, this species is known to inhibit an important diterpene, phytol [56], which has been associated with cytotoxicity [57] and anti-histamine activity [58]. Thus, it is possible that biologically active compounds found in such extracts can trigger anti-inflammatory activity.

Monocytes are immune cells that act in numerous immunological mechanisms, such as replenishment of resident macrophages, production of dendritic cell subsets, and acute and chronic inflammatory responses [59,60]. The cellular activity of these cells involves the production of several molecules responsible for killing of pathogens (H$_2$O$_2$, NO), cellular recruitment (IL-8, CCL2, and CCL3), pro-inflammatory activation (TNF-α, IL-1β, and IL-6), polarization of adaptive immune response (IL-12), regulation of inflammation (IL-10 and TGF-β1), and tissue repair (TGF-β1 and bFGF) [40]. This functional plasticity is crucial for

---

**Fig 3. Immunomodulatory effect of crude leaf extract of \textit{Piper umbellata} on LPS-stimulated human monocytes.**

The results of the controls (medium and ketoprofen, unstimulated and LPS stimulated) were used in the analyses for all extract evaluated. Data are expressed as median ± SEM (pg/mL). ANOVA with Bonferroni post-test; p < 0.05; *: significantly different from placebo treatment, #: significantly different from ketoprofen treatment.

https://doi.org/10.1371/journal.pone.0198682.g003
the efficiency of immune systems. On the contrary, the dysregulation of these cells is observed in several inflammatory diseases, including sepsis [61], atherosclerosis [62], rheumatoid arthritis (RA) [63], and hepatic fibrosis [47]. In general, high counts of monocytes and/or intense production of inflammatory mediators by these cells are hallmarks of these diseases [64]. High levels of TNF-α and IL-6 are associated with worse prognoses in septicemia [65] and progression and worsening of RA [66]. Furthermore, long-term use of steroid and non-steroid anti-inflammatory drugs makes treatment difficult. Therefore, in this study, we evaluated the immunotherapeutic effect of the crude extracts using the in vitro model of LPS-stimulated monocyte cell culture that mimics an inflammatory milieu. The principle of the assay is based on monocyte activation by LPS, the major component of the outer membrane of Gram-negative bacteria. Upon binding of LPS to the CD14 receptor and toll-like receptor 4 (TLR-4) of phagocytes [67], several transmembrane and diverse signal transduction cascades are induced to activate transcription factors of the NF-κB family [68,69]. NF-κB translocates from the cytoplasm to the nucleus where it activates target genes responsible for encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes [55–57]. Although the intracellular pathways were not addressed in this study, it is possible that molecules present in the crude extract interfere with the NF-κB signaling pathway. Some anti-inflammatory agents, such as dexamethasone, prednisone, aspirin, sodium salicylate, sulindac, and sulfasalazine have been associated with the repression, or at least in part, of the NF-κB pathway [70, 71]. Thus, the in vitro model used in this study would be helpful in analyzing new anti-inflammatory targets, as well as the underlying mechanisms.

In conclusion, our results showed promising anti-inflammatory and antifibrotic immunomodulatory properties of crude leaf extracts of *P. gaudichaudianum*, *P. arboreum*, *P. umbellata*, and *P. fuligineum*. The results of the controls (medium and ketoprofen, unstimulated and LPS stimulated) were used in the analyses for all extract evaluated. Data are expressed as median ± SEM (pg/mL). ANOVA with Bonferroni post-test; p < 0.05: * significantly different from placebo treatment, #: significantly different from ketoprofen treatment.

https://doi.org/10.1371/journal.pone.0198682.g004
P. fuligineum, and Peperomia obtusifolia, and provided insights for identification of new target drugs to enhance and/or modulate their effects and/or reduce the side-effects.

Supporting information

S1 Fig. Cytotoxic activity of crude extracts from Piper gaudichaudianum, Piper umbellata, Piper fuligineum, Piper fuligineum, and Peperomia obtusifolia. Peripheral blood mononuclear cells were cultured in the presence of different concentrations of the crude extracts (0.156 to 5.0 mg/mL). Concanavalin A (ConA) was used as positive control (lymphoproliferative response). Medium as added in untreated cells for control culture (CC). Data are expressed as median ± SEM (pg/mL). Repeated measures ANOVA with a Dunnett post-test; p < 0.05; * significantly different from placebo treatment, #: significantly different from ketoprofen treatment. TIF

P. fuligineum, and Peperomia obtusifolia, and provided insights for identification of new target drugs to enhance and/or modulate their effects and/or reduce the side-effects.

Supporting information

S1 Fig. Cytotoxic activity of crude extracts from Piper gaudichaudianum, Piper umbellata, Piper fuligineum, Piper fuligineum, and Peperomia obtusifolia. Peripheral blood mononuclear cells were cultured in the presence of different concentrations of the crude extracts (0.156 to 5.0 mg/mL). Concanavalin A (ConA) was used as positive control (lymphoproliferative response). Medium as added in untreated cells for control culture (CC). Data are expressed as median ± SEM (pg/mL). Repeated measures ANOVA with a Dunnett post hoc test; p < 0.05; * p < 0.05; *** p < 0.01; p < 0.001. (TIF)

Acknowledgments

The authors are grateful to Valéria Alves da Silva for providing helpful technical support with the flow cytometry assays. Fundação de Apoio a Pesquisa do Estado de São Paulo—FAPESP provided financial support (Grant 2016/17627-4).

Author Contributions

Conceptualization: Angela Carolina Finato, Maria Sueli Parreira Arruda, Maysa Furlan, James Venturini.
Formal analysis: Marjorie de Assis Golim, Maria Sueli Parreira Arruda, Maysa Furlan, James Venturini.

Funding acquisition: James Venturini.

Investigation: Angela Carolina Finato, Thais Fernanda Fraga-Silva, Amanda Uliana Carvalho Prati, Amauri Alves de Souza Júnior, Bruna Fonseca Mazzeu, Lidiane Gaspareto Felippe, Rute Alves Pinto, Marjorie de Assis Golim, Maysa Furlan, James Venturini.

Methodology: Angela Carolina Finato, Thais Fernanda Fraga-Silva, Amanda Uliana Carvalho Prati, Amauri Alves de Souza Júnior, Bruna Fonseca Mazzeu, Lidiane Gaspareto Felippe, Rute Alves Pinto, Marjorie de Assis Golim, James Venturini.

Project administration: Angela Carolina Finato, Maria Sueli Parreira Arruda, James Venturini.

Resources: Maria Sueli Parreira Arruda, Maysa Furlan, James Venturini.

Supervision: Maria Sueli Parreira Arruda, James Venturini.

Writing – original draft: Angela Carolina Finato, Maria Sueli Parreira Arruda, Maysa Furlan, James Venturini.

Writing – review & editing: Angela Carolina Finato, Maria Sueli Parreira Arruda, Maysa Furlan, James Venturini.

References

1. Yuncker TG. The Piperaceae of Brazil: 1. Piper-group 1, 2, 3, 4. Hoehnea; 1972.
2. Yuncker TG. The Piperaceae of Brazil, 2: Piper—Group 5; Ottonia; Pothomorphe; Sarcorhachis. Hoehnea; 1973.
3. de Figueiredo A, Sazima M. Pollination Biology of Piperaceae Species in Southeastern Brazil. Ann Bot. 2000; 85: 455–460. https://doi.org/10.1006/anbo.1999.1087
4. Jaramillo MA, Manos PS, Zimmer EA. Phylogenetic Relationships of the Perianthless Piperales: Reconstructing the Evolution of Floral Development. Int J Plant Sci. 2004; 165: 403–416. https://doi.org/10.1086/382803
5. Samain M-S, Vrijdaghs A, Hesse M, Goetghhebuer P, Jiménez Rodríguez F, Stoll A, et al. Verhuellia is a segregate lineage in Piperaceae: more evidence from flower, fruit and pollen morphology, anatomy and development. Ann Bot. 2010; 105: 677–688. https://doi.org/10.1093/aob/mcq031 PMID: 20237114
6. Wanke S, Samain M-S, Vanderschaeve L, Mathieu G, Goetghhebuer P, Neinhuis C. Phylogeny of the genus Peperomia (Piperaceae) inferred from the trnK/matK region (cpDNA). Plant Biol Stuttg Ger. 2006; 8: 93–102. https://doi.org/10.1055/s-2005-873060 PMID: 16435273
7. Quijano-Abril MA, Callejas-Posada R, Miranda-Esquivel DR. Areas of endemism and distribution patterns for Neotropical Piper species (Piperaceae). J Biogeogr. 2006; 33: 1266–1278. https://doi.org/10.1111/j.1365-2699.2006.01501.x
8. Di Stasi LC, Hiruma-Lima CA. Plantas medicinais na Amazônia e na Mata Atlântica. 2nd ed. São Paulo: UNESP; 2002.
9. Vasques da Silva R, Navickiene HMD, Kato MJ, Bolzani V da S, Média Cl, Young MCM, et al. Antifungal amides from Piper arboreum and Piper tuberculatum. Phytochemistry. 2002; 59: 521–527. https://doi.org/10.1016/S0031-9422(01)00431-9 PMID: 11853747
10. Miranda JE, Navickiene HMD, Nogueira-Couto RH, Bortoli SAD, Kato MJ, Bolzani V da S, et al. Susceptibility of Apis mellifera (Hymenoptera: Apidae) to pellitorine, an amide isolated from Piper tuberculatum (Piperaceae). Apidologie. 2003; 34: 409–415. https://doi.org/10.1051/apido:2003036
11. Navickiene HMD, Miranda JE, Bortoli SA, Kato MJ, Bolzani VS, Furlan M. Toxicity of extracts and isobutyramides from Piper tuberculatum: potent compounds with potential for the control of the velvetbean caterpillar, Anticarsia gemmatalis. Pest Manag Sci. 2007; 63: 399–403. https://doi.org/10.1002/ps.1340 PMID: 17329416
12. Martínez RCC, Lago JHG, Albuquerque S, Kato MJ. Trypanocidal tetrahydrafuran lignans from inflorescences of Piper solmsianum. Phytochemistry. 2003; 64: 667–670. https://doi.org/10.1016/S0031-9422(03)00356-X PMID: 12943793

13. Felipe LG, Baldoqui DC, Kato MJ, Bolzani V da S, Guimarães EF, Cicarelli RMB, et al. Trypanocidal tetrahydrafuran lignans from Peperomia blanda. Phytochemistry. 2008; 69: 445–450. https://doi.org/10.1016/j.phytochem.2007.08.012 PMID: 17888465

14. Felipe LG, Batista JM, Baldoqui DC, Nascimento IR, Kato MJ, Bolzani VS, et al. Structure and absolute configuration of a secolignan from Peperomia blanda. Phytochem Lett. 2011; 4: 245–249. https://doi.org/10.1016/j.phytolet.2011.04.007

15. Felipe LG, B Jr JM, Baldoqui DC, Nascimento IR, Kato MJ, He Y, et al. VCD to determine absolute configuration of natural product molecules: secolignans from Peperomia blanda. Org Biomol Chem. 2012; 10: 4208–4214. https://doi.org/10.1039/c2ob25109d PMID: 22543980

16. Benevides PJC, Sartorelli P, Kato MJ. Phenylpropanoids and neolignans from Piper regnellii. Phytochemistry. 1999; 52: 339–343. https://doi.org/10.1016/S0031-9422(99)00177-6

17. da Silva Mota J, Leite AC, Batista Junior JM, Noelí López S, Luz Ambrósio D, Duó Passerini G, et al. In vitro trypanocidal activity of phenolic derivatives from Peperomia obtusifolia. Planta Med. 2009; 75: 620–623. https://doi.org/10.1055/s-0029-1185364 PMID: 19241331

18. Lago JHG, Ramos CS, Casanova DCC, Morandim A de A, Bergamo DCB, Cavalheiro AJ, et al. Benzoic acid derivatives from Piper species and their fungitoxic activity against Cladosporium cladosporioides and C. sphaerospermum. J Nat Prod. 2004; 67: 1783–1788. https://doi.org/10.1021/np030530j PMID: 15568762

19. Batista JM, Lopes AA, Ambrósio DL, Regasini LO, Kato MJ, Bolzani V da S, et al. Natural chromenes and chromone derivatives as potential anti-trypanosomal agents. Biol Pharm Bull. 2008; 31: 538–540. PMID: 18310927

20. Batista JM, Batista ANL, Mota JS, Cass OB, Kato MJ, Bolzani VS, et al. Structure elucidation and absolute stereochemistry of isomeric monoterpene chromane esters. J Org Chem. 2011; 76: 2603–2612. https://doi.org/10.1021/jo1025089 PMID: 21401052

21. Batista JM, Batista ANL, Rinaldo D, Villegas W, Ambrósio DL, Cicarelli RMB, et al. Absolute configuration and selective trypanocidal activity of gaudichaudianic acid enantiomers. J Nat Prod. 2011; 74: 1154–1160. https://doi.org/10.1021/np200085h PMID: 21506530

22. Batista JM, Batista ANL, Kato MJ, Bolzani VS, López SN, Nafie LA, et al. Further monoterpene chromane esters from Peperomia obtusifolia: VCD determination of the absolute configuration of a new diastereomeric mixture. Tetrahedron Lett. 2012; 53: 6051–6054. https://doi.org/10.1016/j.tetlet.2012.08.113

23. Navickiene HMD, Morandim A de A, Alécio AC, Regasini LO, Bergamo DCB, Telascrea M, et al. Composition and antifungal activity of essential oils from Piper aduncum, Piper arboreum and Piper tuberculatum. Quim Nova. 2006; 29: 467–470. https://doi.org/10.1590/S0100-40422006000300012

24. Cícero Bezerra Felipe F, Trajano Sousa Filho J, de Oliveira Souza LE, Alexandre Silveira J, Esdras de Andrade Uchoa D, Rocha Silveira E, et al. Pliptarine, an amide alkaloid from Plipta latum. Quim Nova. 2006; 29: 467–470. https://doi.org/10.1590/S0100-40422006000300012

25. Ee GCL, Lim CM, Lim CK, Rahmani M, Shaari K, Bong CFJ. Alkaloids from Piper sarmentosum and Piper nigrum. Nat Prod Res. 2009; 23: 1416–1423. https://doi.org/10.1080/14786410902757998 PMID: 19699114

26. Marques LMM, da Silva-Junior EA, Gouvea DR, Vessecchi R, Pupo MT, Lopes NP, et al. In vitro metabolism of the alkaloid pliptarine by rat liver microsomes. J Pharm Biomed Anal. 2014; 95: 113–120. https://doi.org/10.1016/j.jpba.2014.02.015 PMID: 17399971

27. Kato MJ, Fulan M. Chemistry and evolution of the Piperaceae: Pure and Applied Chemistry. 2007 79: 529–538.

28. Kijjoa A, Giesbrecht AM, Akisue MK, Gottlieb OR, Gottlieb HE. 4-nerolidylcatechol from Potomorphe umbellata. Planta Med. 1980; 39: 85–87.

29. Perazzo FF, Souza GHB, Lopes W, Cardoso LGV, Carvalho JCT, Nanayakkara NPD, et al. Anti-inflammatory and analgesic properties of water–ethanolic extract from Pothomorphe umbellata (Piperaceae) aerial parts. J Ethnopharmacol. 2005; 99: 215–220. https://doi.org/10.1016/j.jep.2005.02.025 PMID: 15894130

30. Jardim ACG, Igloi Z, Shimizu JF, Santos VAFFM, Felippe LG, Mazzeu BF, et al. Natural compounds isolated from Brazilian plants are potent inhibitors of hepatitis C virus replication in vitro. Antiviral Res. 2015; 115: 39–47. https://doi.org/10.1016/j.antiviral.2014.12.018 PMID: 25557602

31. Wu D, Yu L, Nair MG, De Witt DL, Ramsewak RS. Cyclooxygenase enzyme inhibitory compounds with antioxidant activities from Piper methysticum (kava kava) roots. Phytomedicine. 2002; 9: 41–47. https://doi.org/10.1078/0944-7113-00668 PMID: 11924763
32. Regasini LO, Cotinguiba F, Passerini GD, Bolzani V da S, Cicarelli RMB, Kato MJ, et al. Trypanocidal activity of Piper arboreum and Piper tuberculatum (Piperaceae). Rev Bras Farmacogn. 2009; 19: 199–203. https://doi.org/10.1590/S0102-695X2009000200003

33. Regasini LO, Cotinguiba F, Morandim AA, Kato MJ, Scorzonii L, Mendes-Giannini MJ, et al. Antimicrobial activity of Piper arboreum and Piper tuberculatum (Piperaceae) against opportunistic yeasts. Afr J Biotechnol. 2009; 8: 2866–2870.

34. Luis OR, Cotinguiba F, Siqueira JR, Bolzani VS, Silva DH, Furlan M, et al. Radical scavenging capacity of Piper arboreum and Piper tuberculatum (Piperaceae). Lat Am J Pharm. 2008; 27: 900–3.

35. Jardim ACG, Igloi Z, Shimizu JF, Santos V a. FFM, Felippe LG, Mazzeu BF, et al. Natural compounds isolated from Brazilian plants are potent inhibitors of hepatitis C virus replication in vitro. Antiviral Res. 2015; 115: 39–47. https://doi.org/10.1016/j.antiviral.2014.12.018 PMID: 25557602

36. Wu D, Yu L, Nair MG, DeWitt DL, Ramsewak RS. Cyclooxygenase enzyme inhibitory compounds with antioxidant activities from Piper methysticum (kava kava) roots. Phytomedicine Int J Phytother Phytopharm. 2002; 9: 41–47.

37. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983; 65: 55–63. PMID: 6606682

38. Hamdani DA, Javeed A, Ashraf M, Nazir J, Ghafoor A, Altaf I, et al. In vitro cytotoxic and genotoxic evaluation to ascertain toxicological potential of ketoprofen. Afr J Pharm Pharmacol. 2014; 8: 386–391. https://doi.org/10.5897/AJPP2013.3949

39. Russo M, Teixeira H, Marcondes M, Barbuto J. Superoxide-independent hydrogen peroxide release by activated macrophages. Braz J Med Biol Res Rev Bras Pesqui Medicas E Biol. 1989; 22: 1271–1273.

40. Abbas AK, Lichtman AHH, Pillai S. Cellular and Molecular Immunology. Elsevier Health Sciences; 2014.

41. Neovius M, Arkema EV, Olsson H, Eriksson JK, Kristensson LE, Simard JF, et al. Drug survival on TNF inhibitors in patients with rheumatoid arthritis comparison of adalimumab, etanercept and infliximab. Ann Rheum Dis. 2015; 74: 354–360. https://doi.org/10.1136/annrheumdis-2013-204128 PMID: 24285495

42. Lozo Vukovac E, Lozo M, Mise K, Gudelj I, Puljiz Z, Jurcev-Savicevic A, et al. Bronchoalveolar pH and inflammatory biomarkers in newly diagnosed IPF and GERD patients: a case-control study. Med Sci Monit Int Med J Exp Clin Res. 2014; 20: 255–261. https://doi.org/10.12659/MSM.889800 PMID: 24535066

43. Stidham RW, Lee TCH, Higgins PDR, Deshpande AR, Sussman DA, Singal AG, et al. Systematic review with network meta-analysis: the efficacy of anti-TNF agents for the treatment of Crohn’s disease. Aliment Pharmacol Amp Ther. 2014; 39: 1349–1362. https://doi.org/10.1111/apt.12749 PMID: 24749763

44. Chen Y, Qiao Y, Xu Y, Ling W, Pan Y, Huang Y, et al. Serum TNF-α concentrations in type 2 diabetes mellitus patients and diabetic nephropathy patients: A systematic review and meta-analysis. Immunol Lett. 2017; 186: 52–58. https://doi.org/10.1016/j.imlet.2017.04.003 PMID: 28414180

45. Ley B, Brown KK, Collard HR. Molecular biomarkers in idiopathic pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol. 2014; 307: L681–691. https://doi.org/10.1152/ajplung.00144.2014 PMID: 25260757

46. Carleo A, Bennett D, Rottoli P. Biomarkers in sarcoidosis: the contribution of system biology. Curr Opin Pulm Med. 2016; 22: 509–514. https://doi.org/10.1097/MCP.0000000000000306 PMID: 27428796

47. Hemmann S, Graf J, Roderfeld M, Roeb E. Expression of MMPs and TIMPs in liver fibrosis—a systematic review with special emphasis on anti-fibrotic strategies. J Hepatol. 2007; 46: 955–975. https://doi.org/10.1016/j.jhep.2007.02.003 PMID: 17383048

48. Meng X-M, Tang PM-K, Li J, Lan HY. TGF-β1/Smad signaling in renal fibrosis. Front Physiol. 2015; 6. https://doi.org/10.3389/fphys.2015.00082 PMID: 25852569

49. Lopera D, Naranjo TW, Cruz OG, Restrepo A, Cano LE, Lenzi HL. Structure and topographic dynamics of pulmonary histopathology and local cytokine profiles in Paracoccidioides brasiliensis conidia-infected mice. PLoS Negl Trop Dis. 2011; 5: e1232. https://doi.org/10.1371/journal.pntd.0001232 PMID: 21765962

50. Aganna E, Martinon F, Hawkins PN, Ross JB, Swan DC, Booth DR, et al. Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. Arthritis Rheum. 2002; 46: 2445–2452. https://doi.org/10.1002/art.10509 PMID: 12355493

51. Schildberger A, Rossmannith E, Eichhorn T, Strassl K, Weber V. Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns following Stimulation with Lipopolysaccharide. In: Mediators of Inflammation [Internet]. 2013 [cited 18 Mar 2018]. https://doi.org/10.1155/2013/697972 PMID: 23816743
52. Holanda BBC, Guerra RB, Legendre AO, Almeida DF, Fraga-Silva TFC, Finato A, et al. Thermal, spectroscopic and biological studies on solid ibuprofen complexes of heavy trivalent lanthanides and yttrium. Thermochim Acta. 2017; 647: 47–54. https://doi.org/10.1016/j.tca.2016.11.007

53. Astiz M, Saha D, Lustbader D, Lin R, Rackow E. Monocyte response to bacterial toxins, expression of cell surface receptors, and release of anti-inflammatory cytokines during sepsis. J Lab Clin Med. 1996; 128: 594–600. PMID: 8960643

54. Ganguly S, Mula S, Chattopadhyay S, Chatterjee M. An ethanol extract of Piper betle Linn. mediates its anti-inflammatory activity via down-regulation of nitric oxide. J Pharm Pharmacol. 2007; 59: 711–718. https://doi.org/10.1211/jpp.59.5.0012 PMID: 17524237

55. de Fátima Arrigoni-Blank M, Dmitrieva EG, Franzotti EM, Antonioli AR, Andrade MR, Marchioro M. Anti-inflammatory and analgesic activity of Peperomia pellucida (L.) HBK (Piperaceae). J Ethnopharmacol. 2004; 91: 215–218. https://doi.org/10.1016/j.jep.2003.12.030 PMID: 15120441

56. Pejin B, Kojic V, Bogdanovic G. An insight into the cytotoxic activity of phytol at in vitro conditions. Nat Prod Res. 2014; 28: 2053–2056. https://doi.org/10.1080/14786419.2014.921686 PMID: 24896297

57. Wei L, Wee W, Syamsumir D. Characterization of Anticancer, Antimicrobial, Antioxidant Properties and Chemical Compositions of Peperomia pellucida Leaf Extract. Acta Medica Iranica. 2011; 49: 670. PMID: 22071643

58. Shimizu T, Tomoo T. Anti-inflammatory Constituents of Topically Applied Crude Drugs. V. Constituents and Anti-inflammatory Effect of Aoki, Aucuba japonica THUNB. Biol Pharm Bull. 1994; 17: 665–667. https://doi.org/10.1248/bpb.17.665 PMID: 7920429

59. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood. 2010; 116: e74–e80. https://doi.org/10.1182/blood-2010-02-258558 PMID: 20628149

60. Gordon S, Taylor P. Monocyte and macrophage heterogeneity. Nature reviews Immunology. 2005; 5: 953. https://doi.org/10.1038/nri1733 PMID: 16322748

61. Campos DP, Silva MV, Machado JR, Castellano LR, Rodrigues V, Barata CH. Early-onset neonatal sepsis: cord blood cytokine levels at diagnosis and during treatment. J Pediatr (Rio J). 2010; 86: 509–514.

62. Tabas I, García-Cardeña G, Owens GK. Recent insights into the cellular biology of atherosclerosis. J Cell Biol. 2015; 209: 13–22. https://doi.org/10.1083/jcb.201412052 PMID: 25869663

63. Brennan FM, Maini RN, Feldmann M. Role of pro-inflammatory cytokines in rheumatoid arthritis. Springer Semin Immunopathol. 1998; 20: 133–147. https://doi.org/10.1007/BF00832003 PMID: 9836373

64. Silveira R, Procianoy R. Evaluation of interleukin-6, tumour necrosis factor-α and interleukin-1β for early diagnosis of neonatal sepsis. Acta Paediatrica. 1999; 88: 647–650. https://doi.org/10.1111/j.1651-2227.1999.tb00015.x PMID: 10419250

65. Meadow W, Rudinsky B. Inflammatory mediator and neonatal sepsis. Rarely has so little been known by so many about so much. Clin Perinatol. 1995; 22: 519–536. PMID: 7671549

66. Srirangan S, Choy EH. The Role of Interleukin 6 in the Pathophysiology of Rheumatoid Arthritis. Ther Adv Musculoskelet Dis. 2010; 2: 247–256. https://doi.org/10.1177/1759720X10378372 PMID: 22870451

67. Ziegler-Heitbrock HWL, Ulevitch RJ. CD14: Cell surface receptor and differentiation marker. Immunol Today. 1993; 14: 121–125. https://doi.org/10.1016/0167-5699(93)90212-4 PMID: 7682078

68. Ghosh S, Baltimore D. Activation In Vitro of NF-(kappa)B by Phosphorylation of Its Inhibitor I(kappa)B. Nature. 1990; 344: 678. https://doi.org/10.1038/344678a0 PMID: 2157987

69. Muroi M, Tanamoto K. The Polysaccharide Portion Plays an Indispensable Role in Salmonella Lipopolysaccharide-Induced Activation of NF-κB through Human Toll-Like Receptor 4. Infect Immun. 2002; 70: 6043–6047. https://doi.org/10.1128/IAI.70.11.6043-6047.2002 PMID: 12379680

70. Anstead GM, Chandrasekar B, Zhang Q, Melby PC. Multinutrient undernutrition dysregulates the resident macrophage proinflammatory cytokine network, nuclear factor-κB activation, and nitric oxide production. J Leukoc Biol. 2003; 74: 982–991. https://doi.org/10.1189/jlb.0203064 PMID: 12960263

71. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest. 2001; 107: 135–142. https://doi.org/10.1172/JCI11914 PMID: 11160126