

Research paper

Protective immune response mediated by neutrophils in experimental visceral leishmaniasis is enhanced by IL-32 γ

Rodrigo Saar Gomes^{a,*}, Muriel Vilela Teodoro Silva^a, Milton Adriano Pelli Oliveira^a, Leo A. B. Joosten^b, Fátima Ribeiro-Dias^{a,*}

^a Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia, Brazil

^b Department of Internal Medicine and Radboud Center of Infectious Diseases (RCI), Radboud University Medical Center, Nijmegen, the Netherlands



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ABSTRACT

Neutrophils are important cells in protection against microbial infections including visceral leishmaniasis (VL). It is well known that IL-32 γ increases the protective T helper 17 cell mediated immune response against *Leishmania infantum*. Thus, in this study we evaluated whether IL-32 γ can increase the protective role of neutrophils against VL. In comparison with wild type (WT) mice, transgenic mice for human IL-32 γ (IL-32 γ Tg) presented a higher frequency and absolute number of neutrophils in both spleen and liver after the establishment of *L. infantum* infection. The IL-32 concentrations correlated with neutrophil numbers in the infected tissues. The IL-32 γ -induced recruitment of neutrophils was dependent on IL-17, since inhibition of Th17 T cells generation and IL-17 production with digoxin treatment reversed the effects of IL-32 γ . In murine neutrophils, the presence of IL-32 γ enhanced the phagocytosis of *L. infantum* via CR3. In addition, murine IL-32 γ Tg neutrophils were able to kill *L. infantum* due to the increased production of ROS when compared with WT neutrophils. In fact, IL-32 γ Tg mice lost their ability to control infection by *L. infantum* when neutrophils were depleted. In parallel, treatment of human neutrophils with recombinant IL-32 γ increased phagocytosis and ROS-dependent killing of *L. infantum*, similarly to murine IL-32 γ Tg neutrophils. The data show that IL-32 γ induces neutrophil recruitment to organs affected by VL and increases phagocytosis and killing of *L. infantum* by neutrophils. Together, data indicate the pivotal axis IL-32 γ -Th17-neutrophils to control VL.

1. Introduction

Visceral leishmaniasis (VL) is an infectious-parasitic disease, caused by *Leishmania infantum* in South America, South Europe, Africa and Asia, and *Leishmania donovani* in Asia and Africa. These parasites are transmitted by bites of infected phlebotomine sandflies of the genus *Lutzomyia* or *Phlebotomus*. *L. infantum* and *L. donovani* spread to spleen, liver and bone marrow, causing hepatosplenomegaly, fever, anemia, weight loss, and can be fatal if left untreated. There are an estimated 400,000 new cases and 50,000 deaths by VL annually worldwide [1-3].

Macrophages are the main host cells for *Leishmania* spp. In general, the Th1 immune response is protective in experimental and human leishmaniasis due to the ability of IFN- γ in activating macrophages to increase microbicidal molecules, such as nitric oxide (NO) and reactive oxygen species (ROS) [4-7]. In addition, neutrophils play a fundamental role in the immune response during leishmaniasis [8-11]. Apoptotic-infected neutrophils can act as a “trojan horse”, being phagocytosed

by macrophages, which favors the parasite persistence [8,12]. However, neutrophils are able to phagocytose and kill *Leishmania* by producing microbicidal molecules as ROS and by extracellular neutrophil traps (NETs) [13-19]. Moreover, neutrophils secrete cytokines and chemokines that recruit and/or activate other immune cells during VL [20-23].

Neutropenia appears to be a risk factor for mortality in VL [23,24] and anti-neutrophil treatment increases severity of *L. infantum* and *L. donovani* infection in experimental murine models [11,25-28]. Neutrophil recruitment to spleen and liver seems to be mediated by IL-17, mainly produced by Th17 cells after the infection [22,28-30]. In general, wild type (WT) C57BL/6 mice infected with *L. infantum* present low production of IL-17 [29]. However, we have demonstrated that human IL-32 γ (IL-32 γ increases the Th17 response and IL-17 production in *L. infantum*-infected C57BL/6 mice [31].

IL-32 is a cytokine with nine different isoforms among which IL-32 γ is the most pro-inflammatory. IL-32 γ induces the production of other pro-inflammatory cytokines such as TNF α , IL-6 and IL-1 β in innate

* Corresponding authors.

E-mail addresses: rodrigosaar@ufg.br (R.S. Gomes), fdias@ufg.br (F. Ribeiro-Dias).

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immunity, whereas contributing to polarize the acquired immune response toward a mixed Th1/Th17 profile [32-34]. Although there is no human homologous gene of IL-32 in rodents, murine cells respond to this cytokine [34,35]. The presence of human IL-32 γ in mice (IL-32 γ -transgenic mice, IL-32 γ Tg) is protective in murine VL due to the induction of Th1/IFN- γ and Th17/IL-17 immune response and, consequently, the induction of NO production *in vivo* [31]. Since IL-32 γ induces the production of IL-17 [31,36] and IL-17 is known to induce the influx of protective neutrophils during the established experimental VL [22,29], we hypothesized that the presence of IL-32 γ in mice may increase the protective role of neutrophils during this disease.

In this study, we investigated the role of IL-32 γ in the recruitment of neutrophils into organs affected by *L. infantum* infection in an IL-32 γ Tg mouse model. Furthermore, we evaluated the role of this cytokine in the ability of phagocytosis and killing of *L. infantum* by murine and human neutrophils.

2. Methods

2.1. Ethics statement

All animal procedures were followed in accordance with the guidelines and legislation on ethics of the Brazilian Society of Science in Laboratory Animals (SBCAL) and National Council of Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee in the Use of Animals/CEUA-PRPI-UFG (protocol no. 042/16). The human study was approved by Ethics Committee of the Hospital das Clínicas/Universidade Federal de Goiás (protocol 44.033.514.0.0000.5078). Healthy control subjects who accepted the invitation to participate in the research signed the informed consent form.

2.2. Mice

C57BL/6 mice IL-32 γ Tg were generated as previously described [37]. IL-32 γ Tg and wild-type C57BL/6 (WT) were maintained at the animal facility of Instituto de Patologia Tropical e Saúde Pública/Federal University of Goiás, Brazil, with water and food *ad libitum*. Six to eight-week-old mice were used in the experiments.

2.3. Parasites

L. (L.) infantum (MHOM/BR/74/PP/75) promastigotes were cultured in Grace's Insect Medium (Gibco-Life Technologies) supplemented with heat-inactivated 20% Fetal Calf Serum (FCS, Gibco-Life Technologies), 0.2 mM L-glutamine (Sigma-Aldrich) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich) at 26 °C. Stationary phase promastigotes (5th day of culture) were washed three times with sterile phosphate-buffered saline (PBS), pH 7.4 (1000 g, 10 min, 10 °C), suspended in PBS and quantified in hemocytometer.

2.4. In vivo infection and analysis of neutrophil recruitment

IL-32 γ Tg and WT mice were infected intraperitoneally with 10^7 stationary phase promastigotes of *L. infantum*. After 1, 7, 14 or 28 days of infection, mice were euthanized and the spleen and liver were collected to assess parasite burden by limiting dilution technique, as previously described [38], and neutrophil recruitment. Uninfected mice were used as control (day 0). To evaluate the neutrophil infiltration, spleen and liver fragments were harvested, weighed and macerated to obtain single-cell suspensions in 0.5 mL of PBS/10% FCS. The cells were stained with antibody Alexa Fluor® 594 anti-mouse Ly6G (clone 1A8) and PE anti-mouse CD11b (clone M1/70) or isotype controls (all from eBioscience) for 20 min, at room temperature. The cells were washed with PBS and fixed with 1% paraformaldehyde (Sigma-Aldrich). The neutrophils (CD11b⁺Ly6G⁺ cells) were quantified in a BD Accuri C6 flow cytometer (BD Bioscience), and the data were analyzed using FSC v.4

software (DNS). In some experiments, neutrophils were depleted by intraperitoneal injections of RB6-8C5 monoclonal antibody (200 μ g) one day before infection, on the day of infection, and every 3 days after infection [39]. Mouse IgG was used as control. Neutrophil depletion was confirmed by quantifying CD11b⁺Ly6G⁺ cells in both spleen and liver as described above.

2.5. Quantification of cytokines and in vivo suppression of IL-17

To measure IL-17A and IL-32 concentrations in organs, spleen and liver were harvested, weighed, and macerated in 0.5 mL of PBS/0.1% Triton X-100 (Sigma-Aldrich) with protease inhibitor cocktail (Sigma-Aldrich). Murine IL-17A (BD Biosciences) and human IL-32 (R&D Systems) was evaluated by enzyme-linked immunosorbent assay (ELISA) kits, according to manufacturer's instructions. The results were expressed as picogram of IL-32/mg of tissue. Th17 and IL-17A production was suppressed *in vivo* by intraperitoneal injection of digoxin (40 μ g) every 2 days after infection [40,41].

2.6. Mouse neutrophil isolation

Peritoneal neutrophils were obtained by intraperitoneal injection of 10% thioglycolate (Sigma-Aldrich) as previously described [42]. After 18 h, cells were collected by washing the mouse peritoneum with cold PBS and left to adhere for 2 h on plastic plates (Corning-Costar). Non-adherent cells were collected and neutrophils quantified by flow cytometry, as described above. Neutrophil suspensions of purity >85% were used. Neutrophils (1×10^6 cells/mL) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS (Gibco, Life Technologies), 2 mM L-glutamine, 11 mM sodium bicarbonate, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma-Aldrich).

2.7. L. infantum uptake and control by murine neutrophils

L. infantum promastigotes were stained with carboxyfluorescein succinimidyl ester (CFSE; Sigma-Aldrich), as described before [43]. After 0.5, 1 and 2 h of infection (MOI 5:1), at 36 °C, the infectivity in neutrophils was evaluated in flow cytometry (described above). Frequency of CFSE⁺ cells and mean fluorescence intensity (MFI) were evaluated in a BD Accuri C6 flow cytometer (BD Bioscience). To assess intracellular load of *L. infantum*, neutrophils were infected for 2 h, at 36 °C/5% CO₂, cells were centrifuged at low rotation (200 g/10 min/4 °C) to remove non-internalized parasites and supernatant containing non-internalized promastigotes was discarded. Infected neutrophils were incubated for an additional 20 h. The neutrophil culture medium was replaced, after 2 h or 20 h, by Grace's Insect Medium supplemented as described above. The cell cultures were incubated for 48 h, at 26 °C, in an anaerobic environment, for neutrophils destruction and maintenance/transformation of parasites into flagellated promastigote forms. Viable promastigotes were quantified in hemocytometer after dilution in PBS-0.1% formaldehyde. Alternatively, in some experiments, complement receptor 3 (CR3) was blocked by monoclonal antibody anti-CD11b (M1/70 clone) [44] and ROS production was inhibited by addition of apocynin (10^{-4} M; Sigma-Aldrich) 30 min before infection, and replenished after washings (2 h).

We evaluated the viability of murine neutrophils up to 20 h of incubation by MTT assay [45]. WT neutrophils had an average of 78.12% (± 6.21) and IL-32 γ Tg neutrophils had an average of 81.41% (± 6.32) viability compared to the beginning of the incubation.

2.8. Assessment of neutrophil activities

TNF- α was quantified in the supernatant of neutrophil cultures by ELISA kit (BD Bioscience). Myeloperoxidase (MPO) activity was evaluated by adding tetramethylbenzidine (Sigma-Aldrich) in culture supernatant and measuring optical density values at 450 nm [46]. ROS

production was evaluated by 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen), according to the manufacturer's instructions, and analyzed in a flow cytometer.

2.9. Human neutrophil infection and treatment with IL-32 γ

Polymorphonuclear cells (PMN) from six healthy donors were isolated based on density gradient centrifugation using Ficoll-Paque (GE Healthcare) as previously described [47] and the purity of PMN (>90%) was evaluated by morphological analysis after Giemsa (Merck) staining. PMN cells (1×10^6 cells/mL) were treated with 200 ng/mL recombinant human IL-32 γ (rhIL-32 γ ; R&D System) for 1 h and incubated with *L. infantum* promastigotes (MOI 5:1) for 2 h or 20 h, as described above in "L. infantum uptake and control by murine neutrophils" section of methods. These experiments were performed in the presence of polymyxin B (5 μ g/mL; Sigma-Aldrich) to discard any effect of lipopolysaccharide contamination in rhIL-32 γ batch. *L. infantum* uptake and control and ROS production were evaluated as described above.

2.10. Statistical analysis

Data were expressed as means \pm standard errors of the mean (SEM). Student's *t* test and one-way or two-way analysis of variance (ANOVA) with Bonferroni's posttest were used. The median, individual values and the Mann-Whitney *U* test (unpaired) or Wilcoxon signed-ranks test (paired) were used for nonparametric data. GraphPad Prism v.6 software (San Diego, CA, USA) was used. Level of statistical significance was established at $p < 0.05$.

3. Results

3.1. IL-32 γ induces neutrophil recruitment during experimental visceral leishmaniasis in an IL-17-dependent manner

Since IL-32 γ favors a Th17 immune profile [31] and IL-17 is able to enhance neutrophil recruitment during VL [29,30], it was assessed the number of neutrophils (CD11b⁺Ly6G⁺ cells) in the spleen and liver from

L. infantum-infected IL-32 γ Tg or WT mice (Supplementary Fig. S1). IL-32 γ Tg mice presented a higher percentage of neutrophils from the 7th until 28th day post infection with *L. infantum* compared with WT mice, in both spleen (Fig. 1A) and liver (Fig. 1C). Indeed, the absolute number of neutrophils in spleen (Fig. 1B) and liver (Fig. 1D) were also significantly increased in IL-32 γ Tg mice. Corroborating with the hypothesis that IL-32 γ can contribute to neutrophil recruitment, IL-32 levels in the spleen and liver of infected IL-32 γ Tg mice were correlated with the number of neutrophils on day 14th and 28th of infection (Fig. 2).

Confirming previous data [31], we observed that the production of IL-17A was increased in the spleen and liver (Fig. 3A) of IL-32 γ Tg mice, compared with WT mice, from day 7 (spleen) or day 14 (liver) until day 28 of infection. To assess the participation of IL-17 in IL-32 γ -induced neutrophil recruitment, mice were treated with digoxin to suppress the production of IL-17A (Supplementary Fig. S2). In fact, inhibition of IL-17A production significantly reduced the percentage and absolute number of neutrophils in the spleen (Fig. 3B) and liver (Fig. 3C) of IL-32 γ Tg mice after 14 days (Fig. 3B and C) as well as after 28 days (Fig. 3D and E) of infection with *L. infantum*. Although it was observed a reduction in the recruitment of neutrophils in digoxin-treated infected WT mice (in average 1.99-fold and 2.41-fold decrease; % neutrophils and absolute number of neutrophils, respectively), the effects of IL-17A on the recruitment of neutrophils were exacerbated in the presence of IL-32 γ (in average 2.83-fold and 3.69-fold decrease; % neutrophils and number of neutrophils, respectively).

3.2. IL-32 γ increases *L. infantum* phagocytosis by inflammatory murine neutrophils in a CR3-dependent manner.

Since IL-32 γ increased the recruitment of neutrophils during experimental VL, we decided to investigate whether this cytokine directly alters the functional activity of these cells. To assess the effects of IL-32 γ on the phagocytic capacity of murine neutrophils, thioglycolate-elicited inflammatory neutrophils were incubated with CFSE⁺-*L. infantum* for up to 2 h and parasite uptake was evaluated by flow cytometry (percentage of CFSE⁺ cells and MFI). IL-32 γ Tg neutrophils presented higher uptake of *L. infantum* than WT neutrophils (Fig. 4A and Supplementary Fig. S3).

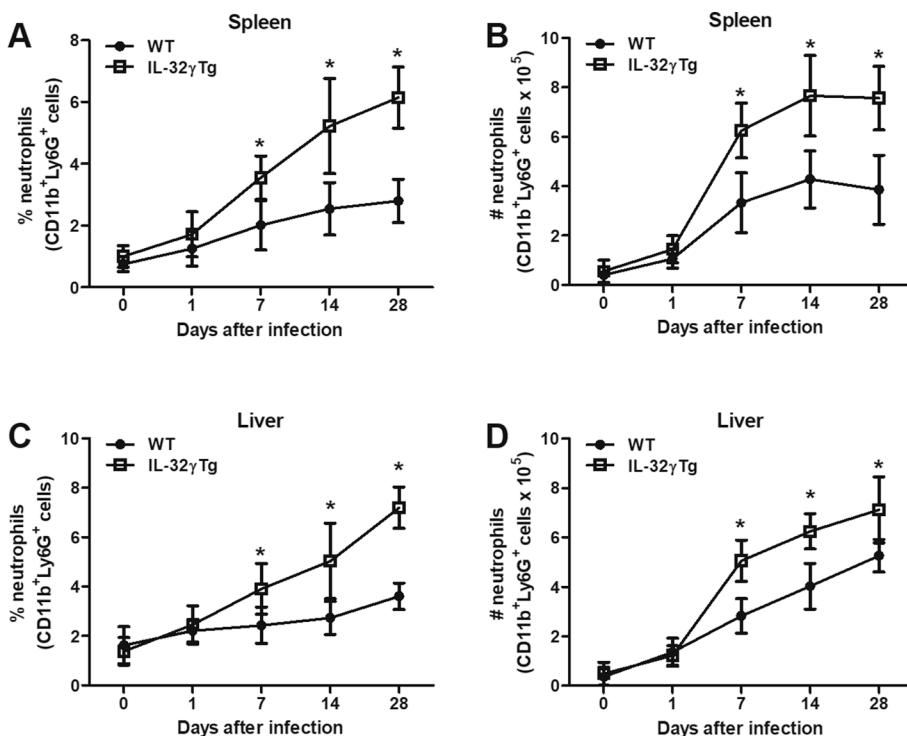


Fig. 1. IL-32 γ increases the recruitment of neutrophils into spleen and liver of *L. infantum*-infected mice. WT and IL-32 γ Tg mice were infected, i.p., with 10^7 *L. infantum* promastigotes for 1, 7, 14 or 28 days. The relative (%) (A and C) and absolute number (B and D) of neutrophils (CD11b⁺Ly6G⁺ cells) were assessed by flow cytometry of spleen (A and B) and liver (C and D) single-cell suspensions. The data show the mean \pm SEM of 9 mice, from 3 independent experiments. * $p < 0.05$ (WT vs. IL-32 γ Tg), by two-way ANOVA/Bonferroni's posttest.

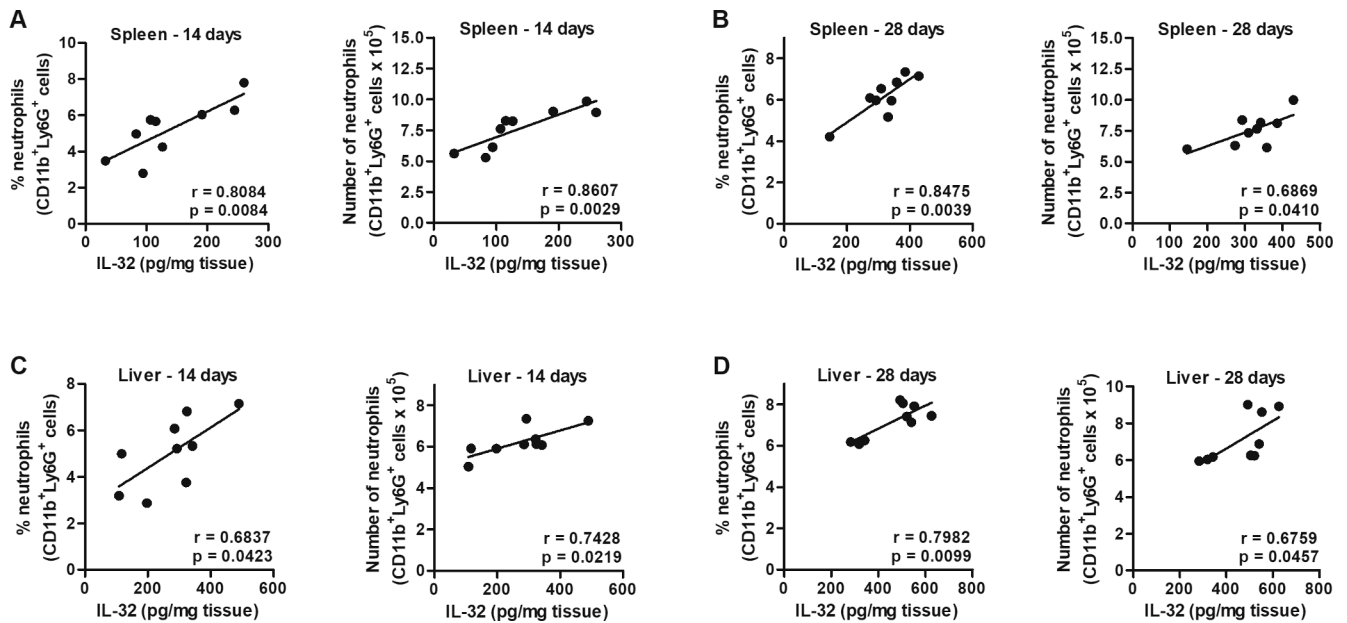


Fig. 2. IL-32 levels correlate with the number of neutrophils recruited during experimental VL. The levels of IL-32, assessed by ELISA, in the (A and B) spleen and (C and D) liver of IL-32 γ Tg mice, were correlated with the percentage and absolute number of neutrophils on 14th or 28th days of infection. The data are represented as individual values ($n = 9$), r and p -value, by Pearson correlation test.

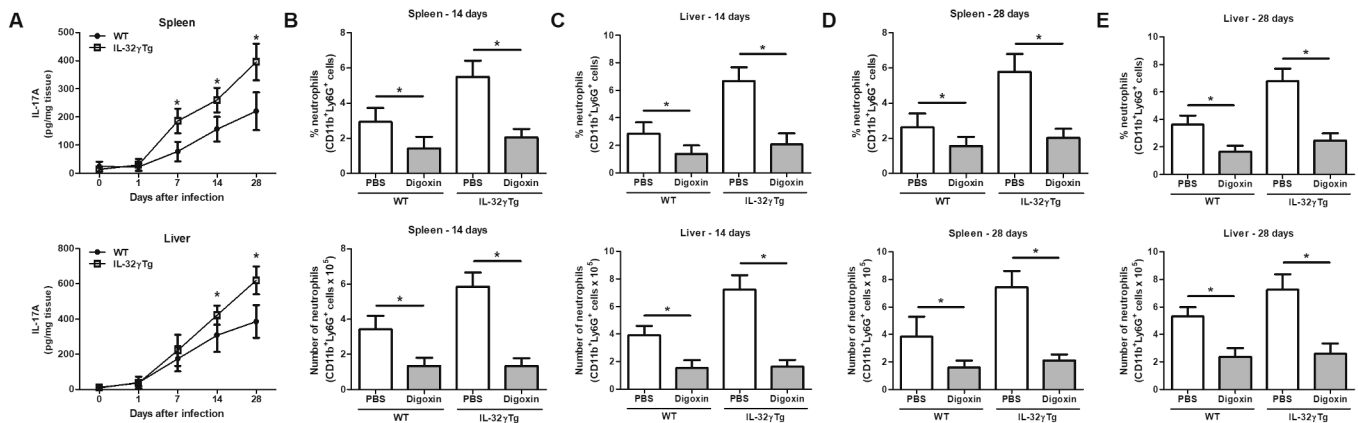


Fig. 3. IL-32 γ -increased recruitment of neutrophils is dependent on IL-17. WT and IL-32 γ Tg mice were infected, i.p., with 10^7 *L. infantum* promastigotes for 1, 7, 14 or 28 days. IL-17A was measured in the (A) spleen (upper panel) and liver (lower panel) lysates by ELISA. Mice were injected i.p. with PBS or digoxin (40 μ g, every 2 days after infection), for IL-17 suppression. (B - E) The percentage and number of neutrophils in spleen and liver of *L. infantum*-infected mice on 14 or 28 days post infection. The data show the mean \pm SEM of 9 mice, from 3 independent experiments. In A, * $p < 0.05$ (WT vs. IL-32 γ Tg), by two-way ANOVA/Bonferroni's posttest. In B - E, * $p < 0.05$ (PBS vs. Digoxin), by Student's t test.

In fact, the number of viable parasites recovered after 2 h of infection was greater in IL-32 γ Tg neutrophil cultures than in WT ones (Fig. 4B).

Further, expression of CR3, one of the main receptors for *Leishmania* spp. uptake by innate immune cells, was evaluated by using fluorescent anti-CD11b antibody. It was observed that IL-32 γ significantly increased the expression of CR3 in uninfected neutrophils compared to neutrophils from WT mice (Fig. 4C). Blocking CR3 with neutralizing anti-CR3 antibody reversed the enhancement of *L. infantum* phagocytosis induced by IL-32 γ (Fig. 4D). In our hands, CR3 blockade had no effect on *L. infantum* uptake by WT neutrophils (Supplementary Fig. S4).

3.3. IL-32 γ potentiates the neutrophil killing capacity of *L. infantum* via TNF α -ROS-MPO axis

In addition to phagocytosis, neutrophils are important to kill *Leishmania* parasites. Therefore, we evaluated the recovery of viable parasites after 2 h and 20 h of infection to assess the control of *L. infantum* by these

cells. While WT neutrophils were not able to control *L. infantum*, IL-32 γ Tg neutrophils reduced the number of viable parasites *in vitro* (Fig. 5A).

One of most important pathway to control parasite in neutrophils is the ROS production [18,48], which can be induced by TNF α [49]. Although *L. infantum* increased TNF α production in both WT and IL-32 γ Tg, after 2 h of infection, in presence of IL-32 γ , the infection induced higher levels of TNF α in neutrophils compared with WT neutrophils after 20 h (Fig. 5B). In accordance, a similar profile of ROS production was detected after exposure of neutrophils to *L. infantum*. Although it was not observed significant effect of IL-32 γ on the percentage of ROS producing neutrophils, the levels of ROS (MFI) were higher in IL-32 γ Tg than in WT neutrophils at 2 h and 20 h of *L. infantum* infection (Fig. 5C).

To confirm the relevance of ROS for the parasite killing, neutrophils were treated with a NADPH-oxidase inhibitor, apocynin (Supplementary Fig. S5). This treatment reversed IL-32 γ -induced *L. infantum* control in murine neutrophils (Fig. 5D). In agreement with those results, activity of

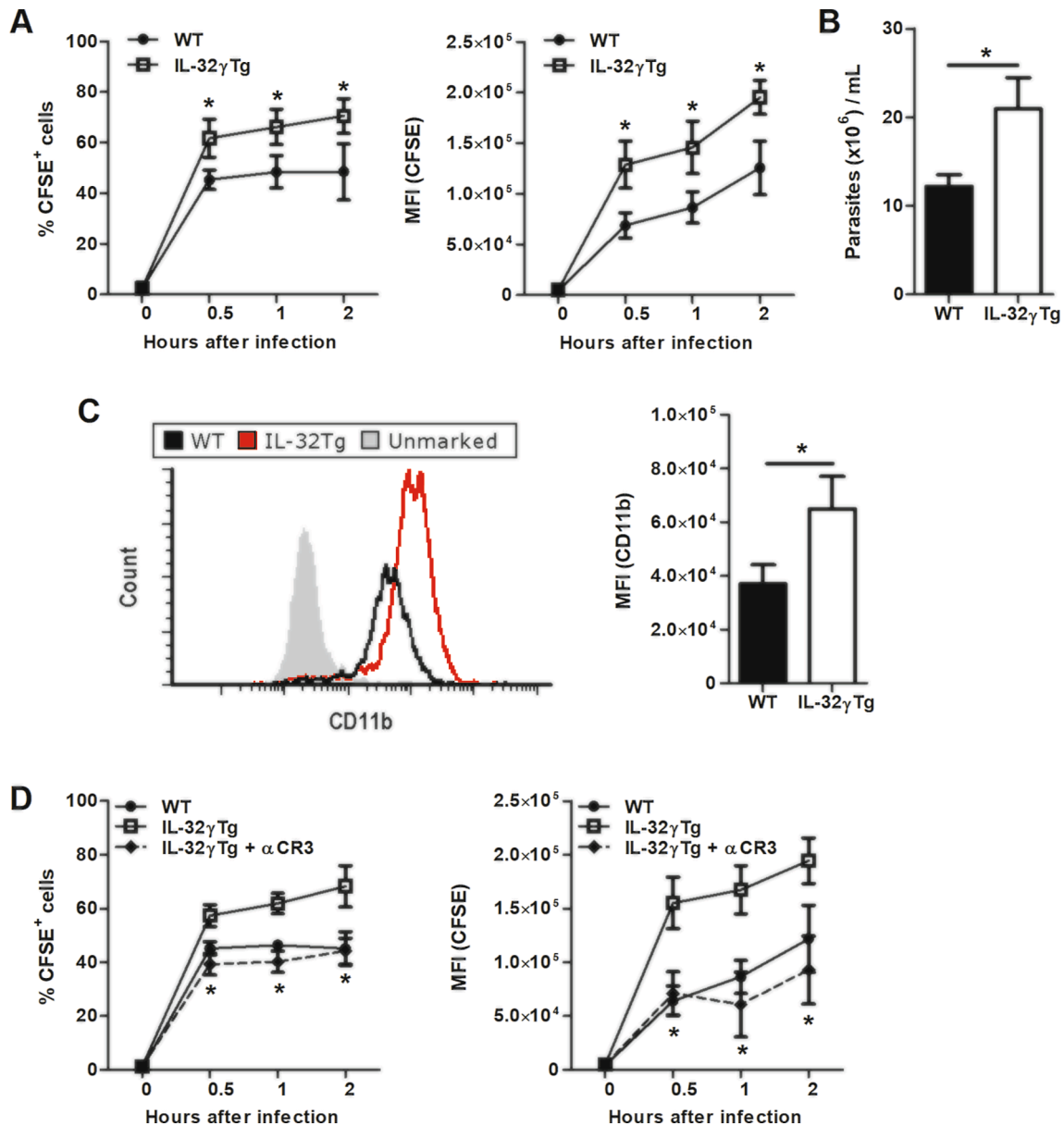


Fig. 4. IL-32 γ increases *L. infantum* phagocytosis by inflammatory murine neutrophils in a CR3-dependent manner. Neutrophils were isolated from the peritoneal cavity of WT and IL-32 γ Tg mice, after 18 h of injection of 10% thioglycolate. (A) Neutrophils were infected with CFSE⁺-*L. infantum* and evaluated by flow cytometry. (B) Number of viable parasites recovered after 2 h of infection. (C) CD11b expression in uninfected neutrophils evaluated by flow cytometry. (D) Neutrophils were treated with anti-CD11b (M1/70 clone, α CR3) and infected with CFSE⁺-*L. infantum* before evaluation by flow cytometry. Data show the mean \pm SEM of 6 mice from 3 independent experiments, performed in duplicate. In A and D, * p < 0.05 (WT vs. IL-32 γ Tg), by two-way ANOVA/Bonferroni's posttest. In B and C, * p < 0.05 (WT vs. IL-32 γ Tg), by Student's *t* test.

myeloperoxidase (MPO), which is an important enzyme in ROS pathway, was higher in IL-32 γ Tg infected neutrophils at 2 h and 20 h of *L. infantum* exposure than WT infected neutrophils (Fig. 5E).

3.4. Neutrophils mediate the IL-32 γ -induced reduction of parasite burden during *in vivo* infection with *L. infantum*

Since IL-32 γ induced neutrophil recruitment during experimental VL and increased *in vitro* phagocytosis and killing of *L. infantum* by these cells, we assessed the participation of neutrophils in IL-32 γ -induced reduction of parasitic burden *in vivo*. Neutrophil depletion (Supplementary Fig. S6) reversed IL-32 γ -induced parasitism control after 14 or 28 days of infection with *L. infantum*, in both spleen and liver (Fig. 6).

3.5. Treatment of human polymorphonuclear cells with recombinant IL-32 γ induces phagocytosis and killing of *L. infantum*

To extend murine neutrophil findings to human neutrophils, we evaluated the effects of IL-32 γ on human PMN cells isolated from the peripheral blood of healthy individuals. Since we were not able to detect IL-32 after incubation of neutrophils with *L. infantum* (data not shown), human PMN cells were treated with rhIL-32 γ . It was observed that treatment with rhIL-32 γ increased the phagocytosis of *L. infantum* by human PMN cells (flow cytometry and number of viable parasites recovered after 2 h of infection) compared with untreated cells (p < 0.05; Fig. 7A, B). Opposite to the increase in phagocytosis, rhIL-32 γ treatment decreased *L. infantum* infection in human PMN cells (2 h vs. 20 h; Fig. 7C). This can be related to the increased production of ROS by infected cells, especially after 20 h of infection (Fig. 7D).

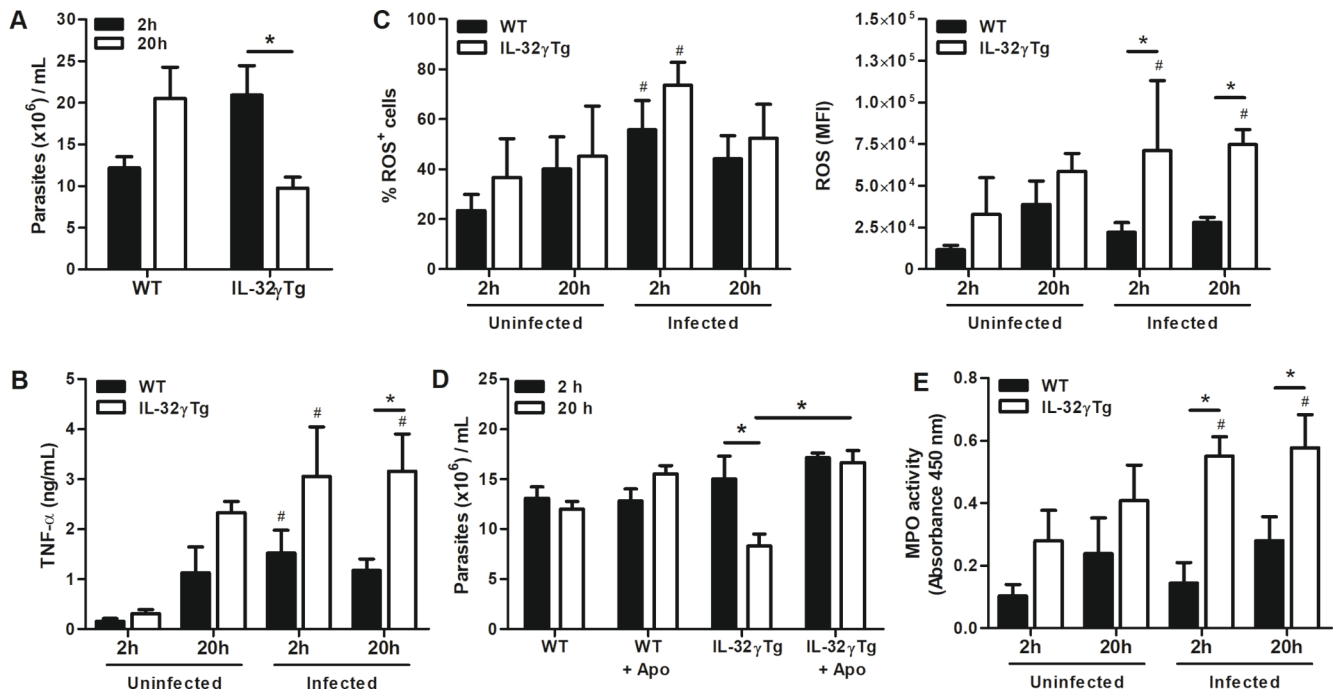


Fig. 5. IL-32 γ activates neutrophils to control *L. infantum* infection via TNF α -ROS-MPO pathway. Neutrophils were isolated from the peritoneal cavity of WT and IL-32 γ Tg mice, after 18 h of injection of 10% thioglycolate and infected with *L. infantum* promastigotes (MOI 5:1) for 2 h or 20 h. (A) Number of viable parasites recovered after 2 h and 20 h of infection. (B) TNF- α production, evaluated by ELISA in supernatant. (C) ROS production evaluated by flow cytometry after incubation with H2DCFDA. (D) Number of viable parasites recovered after 2 h or 20 h of infection in absence or presence of apocynin (10^{-4} M) treatment of neutrophils. (E) MPO activity, evaluated in supernatant incubated with tetramethylbenzidine. The data show the mean \pm SEM of 6 mice, from 3 independent experiments, performed in duplicate. * $p < 0.05$, by one-way ANOVA/Bonferroni's posttest. # $p < 0.05$, by one-way ANOVA/Bonferroni's posttest, compared to the uninfected group, at the same incubation time.

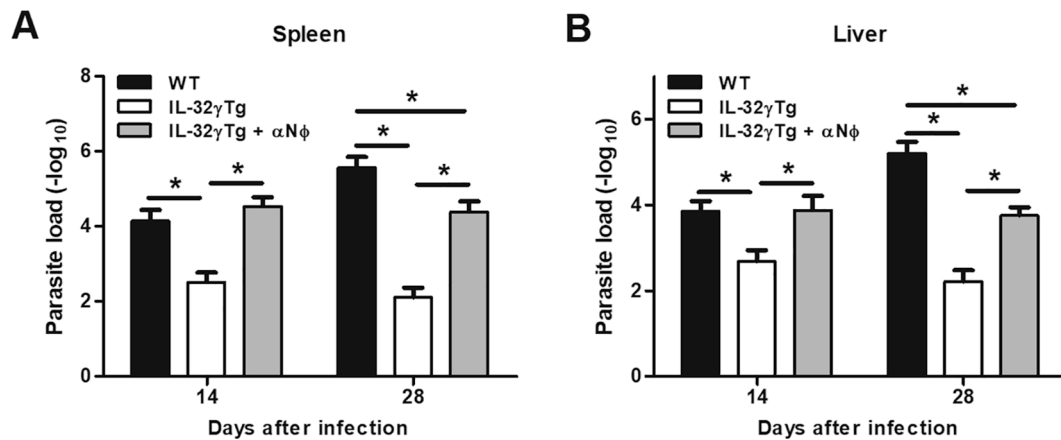


Fig. 6. Neutrophils mediate IL-32 γ -induced reduction of parasite load during *in vivo* infection with *L. infantum*. WT and IL-32 γ Tg mice were infected, i.p., with 10^7 *L. infantum* promastigotes for 14 or 28 days. Neutrophils were depleted with intraperitoneal injections of RB6-8C5 monoclonal antibody. Parasite load was evaluated in (A) spleen and (B) liver. The data show the mean \pm SEM of 6 mice, in 2 independent experiments. * $p < 0.05$, by one-way ANOVA/Bonferroni's posttest.

4. Discussion

We have previously demonstrated that IL-32 γ is a protective cytokine in experimental visceral leishmaniasis. This protection is mediated, at least partially, by the induction of Th1/Th17 by IL-32 γ [31]. In the present study, we showed that the induction of Th17 and the production of IL-17, mediated by IL-32 γ , also induced the recruitment of neutrophils to the *L. infantum* infected organs in a mouse model of VL. Furthermore, the presence of IL-32 γ in murine neutrophils increased the phagocytosis and killing of *L. infantum*. Indeed, IL-32 γ -induced recruitment of neutrophils is crucial for the host protective immune response, since neutrophil depletion reversed IL-32 γ ability to reduce

infection rates *in vivo*.

The protective or permissive role of neutrophils in leishmaniasis appears to be dependent on the species of the parasite, host background and the clinical form of the disease. Neutrophil depletion delays lesion progression and reduces parasite burden in BALB/c mice, while accelerates cutaneous damage in C57BL/6 mice infected with *L. major* [50]. In addition, the presence of neutrophils has protective effects in BALB/c infected with *L. amazonensis*, but the effects are limited in C57BL/6 [39]. The presence of neutrophils may favor tissue injury in tegumentary leishmaniasis [51,52]. Despite different outcome are reached after manipulating neutrophils in different models of murine tegumentary leishmaniasis, it appears that neutrophils play a protective role in VL

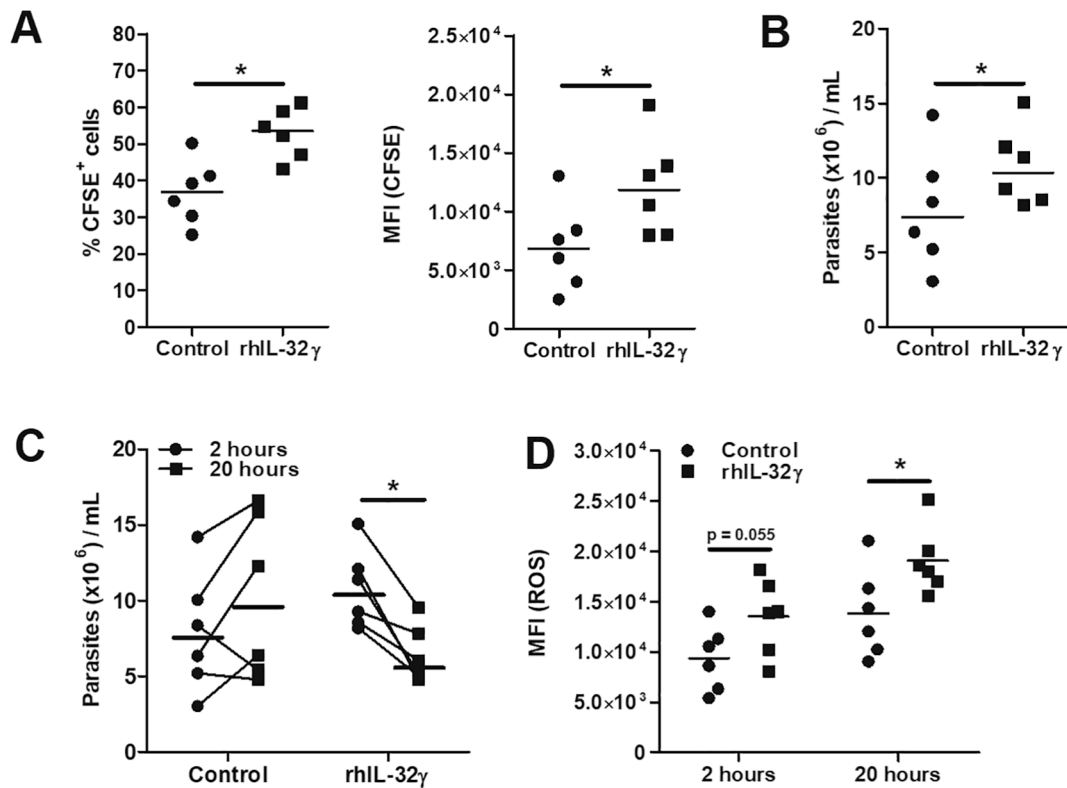


Fig. 7. Treatment of human neutrophils with recombinant IL-32 γ increases phagocytosis, ROS production and killing of *L. infantum*. Polymorphonuclear neutrophils (PMN) from healthy volunteer donors were isolated from peripheral blood based on density gradient centrifugation. PMN cells were treated with 200 ng/mL rhIL-32 γ for 1 h previously to be infected with CFSE⁺-*L. infantum* or non-fluorescent *L. infantum* promastigotes (MOI 5:1) for 2 h or 20 h. (A) % CFSE⁺ cells and MFI. (B) Number of viable parasites recovered after 2 h of infection. (C) Number of viable parasites recovered after 2 h or 20 h of infection. (D) ROS production using H2DCFDA fluorogenic dye. The data show median and individual values of 6 healthy donors. * $p < 0.05$, by Wilcoxon signed-ranks test.

[11,29,53,54]. In fact, we demonstrated here that the presence of neutrophils is crucial for the protection of mice infected with *L. infantum* and IL-32 γ mediates this process.

IL-17 production is essential for neutrophil recruitment, since IL-17 induces the chemoattractive neutrophil CXCL1 [29,55,56]. In addition, IL-17 is known to be involved in host resistance in VL [28,56,57]. However, the IL-17/neutrophil recruitment/resistance axis is impaired in traditional murine models since *L. infantum* infection does not induce the production of large IL-17 levels in C57BL/6 mice affected organs [29,31]. The absence of the IL-32 gene in mouse prevents analyses that better mimic human infection. In fact, we have shown that IL-32, especially IL-32 γ , is fundamental in the control of human infections by different species of *Leishmania*, and the human IL-32 γ Tg C57BL/6 mouse model presents a different profile from cutaneous and VL [31,58-61]. The induction of IL-17 and the role of IL-17 in neutrophil recruitment and protection during VL is now established in C57BL/6 IL-32 γ Tg mouse.

Neutrophils can participate in the control of *Leishmania* infection by the production of ROS, NETs and cytokines/chemokines after parasite phagocytosis [13,23]. Murine and human neutrophils are able to phagocytize *L. infantum* promastigotes [21,25,26,62-64]. In addition, *Leishmania* amastigotes or amastigotes-like presence and replication of amastigotes have been observed within neutrophils [21,63,65]. Here, IL-32 γ increased the uptake of *L. infantum* in neutrophils due to the increased CR3 expression. The role of CR3 in the uptake of *L. major* by neutrophils has already been demonstrated [66] and CR3 is highly expressed on neutrophils, being the main receptor for *Leishmania* in these cells [67]. In addition, IL-32 is known to induce CD11b expression in macrophages [68]. In this study, it was clear that IL-32 increased CR3 on neutrophils at levels that this receptor became very important for *L. infantum* phagocytosis, which was not observed in WT mice.

In addition to the increase of the *L. infantum* phagocytosis, IL-32 γ increased the killing of these parasites within neutrophils, which was mediated mainly by the production of ROS. The release of ROS and TNF α and degranulation of neutrophils are essential for the control of *Leishmania* [42,48,69], but *L. infantum* is capable of interfering with neutrophil activation [21,64]. IL-32 γ clearly increased the activation of murine and human neutrophils, contributing to the killing of *L. infantum* within these cells. Interestingly, the treatment of human neutrophils with recombinant IL-32 γ induces the expression of p38 MAPK (mitogen-activated protein kinase) [70] and p38 MAPK signaling is involved in neutrophil activation, including ROS production and CD11b/CR3 expression [71,72].

Taken together, our data demonstrate that IL-32 γ induces IL-17, which, in turn, induces the recruitment of neutrophils during the establishment of VL. This neutrophil recruitment is essential for the control of *L. infantum* infection observed in the C57BL/6 IL-32 γ Tg mouse model. In addition, we demonstrated that the presence of IL-32 γ increases the uptake and ROS-dependent killing of *L. infantum* in murine and human neutrophils. We have demonstrated mechanistically how IL-32 contributes to the control of VL, in an unprecedented way, increasing the functions of neutrophils.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2021.104449>.

References

- [1] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, J. Jannin, M.D. Boer, M. Kirk, Leishmaniasis worldwide and global estimates of its incidence, *PLoS ONE* 7 (5) (2012) e35671, <https://doi.org/10.1371/journal.pone.0035671>.
- [2] T.S. de Azevedo, C. Lorenz, F. Chiaravalloti-Neto, Risk mapping of visceral leishmaniasis in Brazil, *Rev. Soc. Bras. Med. Trop.* 52 (2019), <https://doi.org/10.1590/0037-8682-0240-2019>.
- [3] H.W. Murray, J.D. Berman, C.R. Davies, N.G. Saravia, Advances in leishmaniasis, *Lancet* 366 (9496) (2005) 1561–1577, [https://doi.org/10.1016/S0140-6736\(05\)67629-5](https://doi.org/10.1016/S0140-6736(05)67629-5).
- [4] P. Kaye, P. Scott, Leishmaniasis: complexity at the host-pathogen interface, *Nat. Rev. Microbiol.* 9 (8) (2011) 604–615, <https://doi.org/10.1038/nrmicro2608>.
- [5] H. Goto, M. das G. Prianti, Immunoactivation and immunopathogeny during active visceral leishmaniasis, *Rev. Inst. Med. Trop. Sao Paulo.* 51 (n.d.) 241–6.
- [6] A. Hailu, D. van Baarle, G.J. Knol, N. Berhe, F. Miedema, P.A. Kager, T cell subset and cytokine profiles in human visceral leishmaniasis during active and asymptomatic or sub-clinical infection with *Leishmania donovani*, *Clin. Immunol.* 117 (2) (2005) 182–191, <https://doi.org/10.1016/j.clim.2005.06.015>.
- [7] K.R. Gantt, T.L. Goldman, M.L. McCormick, M.A. Miller, S.M.B. Jeronimo, E. T. Nascimento, B.E. Britigan, M.E. Wilson, Oxidative Responses of Human and Murine Macrophages During Phagocytosis of *Leishmania chagasi*, *J. Immunol.* 167 (2) (2001) 893–901, <https://doi.org/10.4049/jimmunol.167.2.893>.
- [8] N.C. Peters, J.G. Egen, N. Secundino, A. Debrabant, N. Kimblin, S. Kamhawi, P. Lawyer, M.P. Fay, R.N. Germain, D. Sacks, In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies, *Science* (80-.) 321 (2008) 970–974. doi:10.1126/science.1159194.
- [9] F.L. Ribeiro-Gomes, D. Sacks, The influence of early neutrophil-*Leishmania* interactions on the host immune response to infection, *Front. Cell. Infect. Microbiol.* 2 (2012) 59, <https://doi.org/10.3389/fcimb.2012.00059>.
- [10] R.C. Jochim, C. Teixeira, *Leishmania* commandeers the host inflammatory response through neutrophils, *Trends Parasitol.* 25 (4) (2009) 145–147, <https://doi.org/10.1016/j.pt.2009.01.001>.
- [11] E. McFarlane, C. Perez, M. Charmoy, C. Allenbach, K.C. Carter, J. Alexander, F. Tacchini-Cottier, Neutrophils Contribute to Development of a Protective Immune Response during Onset of Infection with *Leishmania donovani*, *Infect. Immun.* 76 (2) (2008) 532–541, <https://doi.org/10.1128/IAI.01388-07>.
- [12] T. Laskay, G. van Zandbergen, W. Solbach, Neutrophil granulocytes - Trojan horses for *Leishmania* major and other intracellular microbes? *Trends Microbiol.* 11 (5) (2003) 210–214, [https://doi.org/10.1016/S0966-842X\(03\)00075-1](https://doi.org/10.1016/S0966-842X(03)00075-1).
- [13] B.P. Hurrell, I.B. Regli, F. Tacchini-Cottier, Different *Leishmania* Species Drive Distinct Neutrophil Functions, *Trends Parasitol.* 32 (5) (2016) 392–401, <https://doi.org/10.1016/j.pt.2016.02.003>.
- [14] A.B. Guimarães-Costa, M.T.C. Nascimento, G.S. Froment, R.P.P. Soares, F. N. Morgado, F. Conceicao-Silva, E.M. Saraiva, *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps, *Proc. Natl. Acad. Sci. U. S. A.* 106 (16) (2009) 6748–6753, <https://doi.org/10.1073/pnas.0900226106>.
- [15] F. Wei, W. Gong, J. Wang, Y. Yang, J. Liu, Y. Wang, J. Cao, Role of the lipoxin A4 receptor in the development of neutrophil extracellular traps in *Leishmania* infantum infection, *Parasites Vectors* 12 (1) (2019), <https://doi.org/10.1186/s13071-019-3530-8>.
- [16] N.C. Rochael, A.B. Guimarães-Costa, M.T.C. Nascimento, T.S. DeSouza-Vieira, M. P. Oliveira, L.F. Garcia e Souza, M.F. Oliveira, E.M. Saraiva, Classical ROS-dependent and early/rapid ROS-independent release of Neutrophil Extracellular Traps triggered by *Leishmania* parasites, *Sci. Rep.* 5 (1) (2016), <https://doi.org/10.1038/srep18302>.
- [17] L.G. Gardinassi, T.S. DeSouza-Vieira, N.O. da Silva, G.R. Garcia, V.M. Borges, R.N. S. Campos, R.P. de Almeida, I.K.F. de Miranda Santos, E.M. Saraiva, Molecular signatures of neutrophil extracellular traps in human visceral leishmaniasis, *Parasites Vectors* 10 (1) (2017), <https://doi.org/10.1186/s13071-017-2222-5>.
- [18] M.B.H. Carneiro, E.H. Roma, A.J. Ranson, N.A. Doria, A. Debrabant, D.L. Sacks, L. Q. Vieira, N.C. Peters, NOX2-Derived Reactive Oxygen Species Control Inflammation during *Leishmania amazonensis* Infection by Mediating Infection-Induced Neutrophil Apoptosis, *J. Immunol.* 200 (1) (2018) 196–208, <https://doi.org/10.4049/jimmunol.1700899>.
- [19] E.H. Roma, J.P. Macedo, G.R. Goes, J.L. Gonçalves, W.d. Castro, D. Cisalpino, L. Q. Vieira, Impact of reactive oxygen species (ROS) on the control of parasite loads and inflammation in *Leishmania amazonensis* infection, *Parasites Vectors* 9 (1) (2016), <https://doi.org/10.1186/s13071-016-1472-y>.
- [20] C. Tecchio, M.A. Cassatella, Neutrophil-derived chemokines on the road to immunity, *Semin. Immunol.* 28 (2) (2016) 119–128, <https://doi.org/10.1016/j.smim.2016.04.003>.
- [21] C.S. Marques, L.F.D. Passero, I. Vale-Gato, A. Rodrigues, O.R. Rodrigues, C. Martins, I. Correia, A.M. Tomás, G. Alexandre-Pires, M.H. Ferronha, G. M. Santos-Gomes, New insights into neutrophil and *Leishmania* infantum in vitro immune interactions, *Comp. Immunol. Microbiol. Infect. Dis.* 40 (2015) 19–29, <https://doi.org/10.1016/j.cimid.2015.03.003>.
- [22] S. da C. Gonçalves-de-Albuquerque, R. Pessoa-e-Silva, L.A.M. Trajano-Silva, T.C. de Goes, R.C.S. de Moraes, C.N. da C. Oliveira, V.M.B. de Lorena, M. de Paiva-Cavalcanti, The Equivocal Role of Th17 Cells and Neutrophils on Immunopathogenesis of Leishmaniasis, *Front. Immunol.* 8 (2017) 1437. doi: 10.3389/fimmu.2017.01437.
- [23] E.D. Carlsen, Y. Liang, T.R. Shellet, D.H. Walker, P.C. Melby, L. Soong, Permissive and protective roles for neutrophils in leishmaniasis, *Clin. Exp. Immunol.* 182 (2) (2015) 109–118, <https://doi.org/10.1111/cei.2015.182.issue-210.1111/cei.12674>.
- [24] C.H.N. Costa, G.L. Werneck, D.L. Costa, T.A. Holanda, G.B. Aguiar, A.S. Carvalho, J.C. Cavalcanti, L.S. Santos, Is severe visceral leishmaniasis a systemic inflammatory response syndrome? A case control study, *Rev. Soc. Bras. Med. Trop.* 43 (4) (2010) 386–392, <https://doi.org/10.1590/S0037-86822010000400010>.
- [25] C.J. Thalhofer, Y. Chen, B. Sudan, L. Love-Homan, M.E. Wilson, J.F. Urban, Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan *leishmania infantum* chagasi, *Infect. Immun.* 79 (1) (2011) 108–117, <https://doi.org/10.1128/IAI.00338-10>.
- [26] D. Rousseau, S. Demartino, B. Ferrua, J.F. Michiels, F. Anjuère, K. Fragaki, Y. Le Fichoux, J. Kubar, In vivo involvement of polymorphonuclear neutrophils in *Leishmania* infantum infection, *BMC Microbiol.* 1 (2001) 1–7, <https://doi.org/10.1186/1471-2180-1-17>.
- [27] S.C. Smelt, S.E.J. Cotterell, C.R. Engwerda, P.M. Kaye, B Cell-Deficient Mice Are Highly Resistant to *Leishmania donovani* Infection, but Develop Neutrophil-Mediated Tissue Pathology, *J. Immunol.* 164 (7) (2000) 3681–3688, <https://doi.org/10.4049/jimmunol.164.7.3681>.
- [28] C. Terrazas, S. Varikuti, J. Kimble, E. Moretti, P.N. Boyaka, A.R. Satoskar, IL-17A promotes susceptibility during experimental visceral leishmaniasis caused by *Leishmania donovani*, *FASEB J.* 30 (3) (2016) 1135–1143, <https://doi.org/10.1096/fsb.2015.30.310.1096.fj.15-277202>.
- [29] G.F.S. Quirino, M.S.L. Nascimento, M. Davoli-Ferreira, L.A. Sacramento, M.H. F. Lima, R.P. Almeida, V. Carregaro, J.S. Silva, J.A. Appleton, Interleukin-27 (IL-27) mediates susceptibility to visceral leishmaniasis by suppressing the IL-17-neutrophil response, *Infect. Immun.* 84 (8) (2016) 2289–2298, <https://doi.org/10.1128/IAI.00283-16>.
- [30] L.A. Tesmer, S.K. Lundy, S. Sarkar, D.A. Fox, Th17 cells in human disease, *Immunol. Rev.* 223 (2008) 87–113, <https://doi.org/10.1111/j.1600-065X.2008.00628.x>.
- [31] R.S. Gomes, M.V.T. Silva, J.C. dos Santos, C. van Linge, J.M. Reis, M.M. Teixeira, S. A. Pinto, M.L. Dorta, X. Bai, E.D. Chan, C.A. Dinarello, M.A.P. Oliveira, L.A. B. Joosten, F. Ribeiro-Dias, J.A. Appleton, Human interleukin-32γ plays a protective role in an experimental model of visceral leishmaniasis in mice, *Infect. Immun.* 86 (5) (2018), <https://doi.org/10.1128/IAI.00796-17>.
- [32] J.-W. Kang, Y.S. Park, D.H. Lee, M.S. Kim, Y. Bak, S.Y. Ham, S.H. Park, H. Kim, J. H. Ahn, J.T. Hong, D.-Y. Yoon, Interaction network mapping among IL-32 isoforms, *Biochimie* 101 (2014) 248–251, <https://doi.org/10.1016/j.biochi.2014.01.013>.
- [33] F. Ribeiro-Dias, R. Saar Gomes, L.L. de Lima Silva, J.C. dos Santos, L.A.B. Joosten, Interleukin 32: A novel player in the control of infectious diseases, *J. Leukoc. Biol.* 101 (1) (2017) 39–52, <https://doi.org/10.1189/jlb.4RU0416-175RR>.
- [34] S.-H. Kim, S.-Y. Han, T. Azam, D.-Y. Yoon, C.A. Dinarello, Interleukin-32: A cytokine and inducer of TNFα, *Immunity* 22 (1) (2005) 131–142, <https://doi.org/10.1016/j.immuni.2004.12.003>.
- [35] L.A.B. Joosten, M.G. Netea, S.-H. Kim, D.-Y. Yoon, B. Oppers-Walgreen, T.R. D. Radstake, P. Barrera, F.A.J. van de Loo, C.A. Dinarello, W.B. van den Berg, IL-32, a proinflammatory cytokine in rheumatoid arthritis, *Proc. Natl. Acad. Sci. U. S. A.* 103 (9) (2006) 3298–3303, <https://doi.org/10.1073/pnas.0511233103>.
- [36] M.Y.M.Y. Jung, M.H. Son, S.H. Kim, D. Cho, T.S. Kim, IL-32γ induces the maturation of dendritic cells with Th1- and Th17-polarizing ability through enhanced IL-12 and IL-6 production, *J. Immunol.* 186 (2011) 6848–6859, <https://doi.org/10.4049/jimmunol.1003996>.
- [37] J. Choi, S. Bae, J. Hong, S. Ryoo, H. Jhun, K. Hong, D. Yoon, S. Lee, E. Her, W. Choi, J. Kim, T. Azam, C.A. Dinarello, S. Kim, Paradoxical effects of constitutive human IL-32{γ} in transgenic mice during experimental colitis, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 21082–21086, <https://doi.org/10.1073/pnas.1015418107>.
- [38] S.M.d. Souza-Neto, C.M. Carneiro, L.Q. Vieira, L.C.C. Afonso, *Leishmania braziliensis*: Partial control of experimental infection by interleukin-12 p40 deficient mice, *Mem. Inst. Oswaldo Cruz.* 99 (3) (2004) 289–294, <https://doi.org/10.1590/S0074-02762004000300009>.
- [39] L.M.A. Sousa, M.B.H. Carneiro, M.E. Resende, L.S. Martins, L.M. dos Santos, L. G. Vaz, P.S. Mello, D.M. Mosser, M.A.P. Oliveira, L.Q. Vieira, Neutrophils have a protective role during early stages of *Leishmania amazonensis* infection in BALB/c mice, *Parasite Immunol.* 36 (1) (2014) 13–31, <https://doi.org/10.1111/pim.2013.36.issue-110.1111/pim.12078>.
- [40] M.-A. Hartley, E. Bourreau, M. Rossi, P. Castiglioni, R.O. Eren, F. Prevel, P. Couppié, S.M. Hickerson, P. Launois, S.M. Beverley, C. Ronet, N. Fasel, I. Müller, Leishmanivirus-Dependent Metastatic Leishmaniasis Is Prevented by Blocking IL-17A, *PLOS Pathog.* 12 (9) (2016) e1005852, <https://doi.org/10.1371/journal.ppat.1005852>.

- [41] J.R. Huh, M.W.L. Leung, P. Huang, D.A. Ryan, M.R. Krout, R.R.V. Malapaka, J. Chow, N. Manel, M. Ciofani, S.V. Kim, A. Cuesta, F.R. Santori, J.J. Lafaille, H. E. Xu, D.Y. Gin, F. Rastinejad, D.R. Littman, Digoxin and its derivatives suppress T H17 cell differentiation by antagonizing ROR γ 3t activity, *Nature* 472 (2011) 486–490, <https://doi.org/10.1038/nature09978>.
- [42] S.A.C. Falcão, T. Weinkopff, B.P. Hurrell, F.S. Celes, R.P. Curvelo, D.B. Prates, A. Barral, V.M. Borges, F. Tacchini-Cottier, C.I. de Oliveira, S. Kamhawi, Exposure to *Leishmania braziliensis* Triggers Neutrophil Activation and Apoptosis, *PLoS Negl. Trop. Dis.* 9 (3) (2015) e0003601, <https://doi.org/10.1371/journal.pntd.0003601>.
- [43] R. Gonçalves, E.R. Vieira, M.N. Melo, K.J. Gollob, D.M. Mosser, W.L. Tafuri, A sensitive flow cytometric methodology for studying the binding of *L. chagasi* to canine peritoneal macrophages, *BMC Infect. Dis.* 5 (1) (2005), <https://doi.org/10.1186/1471-2334-5-39>.
- [44] A. Cooper, H. Rosen, J.M. Blackwell, Monoclonal antibodies that recognize distinct epitopes of the macrophage type three complement receptor differ in their ability to inhibit binding of *Leishmania* promastigotes harvested at different phases of their growth cycle, *Immunology* 65 (1988) 511–514 (accessed March 11, 2020), <http://www.ncbi.nlm.nih.gov/pubmed/3065216>.
- [45] M. T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods.* 65 (1983) 55–63, [https://doi.org/10.1016/0022-2759\(83\)90303-4](https://doi.org/10.1016/0022-2759(83)90303-4).
- [46] P.C. Andrews, N.I. Krinsky, Quantitative determination of myeloperoxidase using tetramethylbenzidine as substrate, *Anal. Biochem.* 127 (2) (1982) 346–350, [https://doi.org/10.1016/0003-2697\(82\)90185-3](https://doi.org/10.1016/0003-2697(82)90185-3).
- [47] D.B. Kuhns D.A.L. Priel J. Chu K.A. Zarembler. in: *Curr. Protoc. Immunol., Isolation and Functional Analysis of Human Neutrophils 2015* John Wiley & Sons Inc Hoboken, NJ, USA pp. 7.23.1-7.23.16. 10.1002/0471142735.im0723s111.
- [48] R. Ricci-Azevedo, A.F. Oliveira, M.C.A.V. Conrado, F.C. Carvalho, M.C. Roque-Barreira, R. Correa-Oliveira, Neutrophils Contribute to the Protection Conferred by ArtinM against Intracellular Pathogens: A Study on *Leishmania major*, *PLoS Negl. Trop. Dis.* 10 (4) (2016) e0004609, <https://doi.org/10.1371/journal.pntd.0004609>.
- [49] K. Futosi, S. Fodor, A. Mócsai, Neutrophil cell surface receptors and their intracellular signal transduction pathways, *Int. Immunopharmacol.* 17 (3) (2013) 638–650, <https://doi.org/10.1016/j.intimp.2013.06.034>.
- [50] F. Tacchini-Cottier, C. Zweifel, Y. Belkaid, C. Mukankundiye, M. Vasei, P. Launois, G. Milon, J.A. Louis, An Immunomodulatory Function for Neutrophils During the Induction of a CD4 + Th2 Response in BALB/c Mice Infected with *Leishmania major*, *J. Immunol.* 165 (5) (2000) 2628–2636, <https://doi.org/10.4049/jimmunol.165.5.2628>.
- [51] V.S. Boaventura, C.S. Santos, C.R. Cardoso, J. de Andrade, W.L.C. Dos Santos, J. Clarêncio, João.S. Silva, V.M. Borges, M. Barral-Netto, C.I. Brodskyn, A. Barral, Human mucosal leishmaniasis: Neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines, *Eur. J. Immunol.* 40 (10) (2010) 2830–2836, <https://doi.org/10.1002/eji.200940115>.
- [52] B.P. Hurrell, S. Schuster, E. Grün, M. Coutaz, R.A. Williams, W. Held, B. Malissen, M. Malissen, S. Yousefi, H.-U. Simon, A.J. Müller, F. Tacchini-Cottier, I. Müller, Rapid Sequestration of *Leishmania mexicana* by Neutrophils Contributes to the Development of Chronic Lesion, *PLoS Pathog.* 11 (5) (2015) e1004929, <https://doi.org/10.1371/journal.ppat.1004929>.
- [53] L. Sacramento, S.C. Trevelin, M.S. Nascimento, D.S. Lima-Júnior, D.L. Costa, R. P. Almeida, F.Q. Cunha, João.S. Silva, V. Carregaro, J.A. Appleton, Toll-like receptor 9 signaling in dendritic cells regulates neutrophil recruitment to inflammatory foci following *Leishmania infantum* infection, *Infect. Immun.* 83 (12) (2015) 4604–4616, <https://doi.org/10.1128/IAI.00975-15>.
- [54] P. Yurdakul, J. Dalton, L. Beattie, N. Brown, S. Erguven, A. Maroof, P.M. Kaye, Compartment-specific remodeling of splenic micro-architecture during experimental visceral leishmaniasis, *Am. J. Pathol.* 179 (1) (2011) 23–29, <https://doi.org/10.1016/j.ajpath.2011.03.009>.
- [55] S. Lopez Kostka, S. Dinges, K. Griewank, Y. Iwakura, M.C. Udey, E. von Stebut, IL-17 Promotes Progression of Cutaneous Leishmaniasis in Susceptible Mice, *J. Immunol.* 182 (5) (2009) 3039–3046, <https://doi.org/10.4049/jimmunol.0713598>.
- [56] M.G.R. Pitta, A. Romano, S. Cabantous, S. Henri, A. Hammad, B. Kouriba, L. Argiro, M. el Kheir, B. Bucheton, C. Mary, S.H. El-Safi, A. Dessen, IL-17 and IL-22 are associated with protection against human kala azar caused by *Leishmania donovani*, *J. Clin. Invest.* 119 (2009) 2379–2387, <https://doi.org/10.1172/JCI38813>.
- [57] G.A.G. Cezário, L.R.C.de Oliveira, E. Peresi, V.C. Nicolette, J. Poletti, C.R. Gonçalves.de Lima, M. Gatto, S.A. Calvi, Analysis of the expression of toll-like receptors 2 and 4 and cytokine production during experimental leishmania chagasi infection, *Mem. Inst. Oswaldo Cruz.* 106 (5) (2011) 573–583, <https://doi.org/10.1590/S0074-02762011000500010>.
- [58] Jéssica.C. dos Santos, B. Heinhuis, R.S. Gomes, M.S.M.A. Damen, F. Real, R. A. Mortara, S.T. Keating, C.A. Dinarello, L.A.B. Joosten, Fátima Ribeiro-Dias, H.da. C. Santiago, Cytokines and microbicidal molecules regulated by IL-32 in THP-1-derived human macrophages infected with New World *Leishmania* species, *PLoS Negl. Trop. Dis.* 11 (2) (2017) e0005413, <https://doi.org/10.1371/journal.pntd.0005413>.
- [59] Hélio Galdino, Anetícia.E. Maldaner, Lívia.L. Pessoni, F.M. Soriani, L.Inácia.de. Araújo. Pereira, Sebastião.A. Pinto, F.B. Duarte, C.M. Gomes, A.K.A. Fleuri, M. L. Dorta, M.A.P. de Oliveira, M.M. Teixeira, A.C. Batista, L.A.B. Joosten, L. Q. Vieira, Fátima Ribeiro-Dias, Interleukin 32 γ (IL-32 γ) is highly expressed in cutaneous and mucosal lesions of American Tegumentary Leishmaniasis patients: association with tumor necrosis factor (TNF) and IL-10, *BMC Infect. Dis.* 14 (1) (2014), <https://doi.org/10.1186/1471-2334-14-249>.
- [60] R.S. Gomes, M.V.T. Silva, J.C. dos Santos, L.L. de Lima Silva, A.C. Batista, J. R. Machado, M.M. Teixeira, M.L. Dorta, M.A.P. de Oliveira, C.A. Dinarello, L.A. B. Joosten, F. Ribeiro-Dias, IL-32 γ promotes the healing of murine cutaneous lesions caused by *Leishmania braziliensis* infection in contrast to *Leishmania amazonensis*, *Parasit. Vectors.* 10 (2017) 336, <https://doi.org/10.1186/s13071-017-2268-4>.
- [61] J.C. Dos Santos, V.B.L. Quixabeira, M.V.T. Silva, M.S.M.A. Damen, K. Schraa, M. Jaeger, M. Oosting, S.T. Keating, M.L. Dorta, S.A. Pinto, F.B. Duarte, L. Inácia de Araújo Pereira, M.G. Netea, F. Ribeiro-Dias, L.A.B. Joosten, Genetic variation in interleukin-32 influence the immune response against new world leishmania species and susceptibility to american tegumentary leishmaniasis, *PLoS Negl. Trop. Dis.* 14 (2020), <https://doi.org/10.1371/journal.pntd.0008029>.
- [62] L.A. Sacramento, J.L. da Costa, M.H.F. de Lima, P.A. Sampaio, R.P. Almeida, F. Q. Cunha, J.S. Silva, V. Carregaro, Toll-like receptor 2 is required for inflammatory process development during *Leishmania infantum* infection, *Front. Microbiol.* 8 (2017), <https://doi.org/10.3389/fmicb.2017.00262>.
- [63] A. Valério-Bolas, M. Pereira, G. Alexandre-Pires, D. Santos-Mateus, A. Rodrigues, M. Rafael-Fernandes, A. Gabriel, F. Passero, G. Santos-Gomes, Intracellular and extracellular effector activity of mouse neutrophils in response to cutaneous and visceral *Leishmania* parasites, *Cell. Immunol.* 335 (2019) 76–84, <https://doi.org/10.1016/j.cellimm.2018.11.003>.
- [64] D. RE, T. CJ, W. ME, Infection and Activation of Human Neutrophils with Fluorescent *Leishmania infantum*, *J. Immunol. Tech. Infect. Dis.* 5 (3) (2016), <https://doi.org/10.4172/2329-954110.4172/2329-9541.1000146>.
- [65] B.P. Hurrell, M. Beaumann, S. Heyde, I.B. Regli, A.J. Müller, F. Tacchini-Cottier, Frontline Science: *Leishmania mexicana* amastigotes can replicate within neutrophils, *J. Leukoc. Biol.* 102 (5) (2017) 1187–1198, <https://doi.org/10.1189/jlb.4HI0417-158R>.
- [66] H. Laufs, K. Müller, J. Fleischer, N. Reiling, N. Jahnke, J.C. Jensenius, W. Solbach, T. Laskay, Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors, *Infect. Immun.* 70 (2) (2002) 826–835, <https://doi.org/10.1128/IAI.70.2.826-835.2002>.
- [67] N. Ueno, M.E. Wilson, Receptor-mediated phagocytosis of *Leishmania*: Implications for intracellular survival, *Trends Parasitol.* 28 (8) (2012) 335–344, <https://doi.org/10.1016/j.pt.2012.05.002>.
- [68] H.-J. Jeong, S.-Y. Nam, H.-A. Oh, N.-R. Han, Y.-S. Kim, P.-D. Moon, S.-Y. Shin, M.-H. Kim, H.-M. Kim, Interleukin-32-induced thymic stromal lymphopoietin plays a critical role in macrophage differentiation through the activation of caspase-1 in vitro, *Arthritis Res. Ther.* 14 (6) (2012) R259, <https://doi.org/10.1186/ar4104>.
- [69] L. Afonso, Valéria.M. Borges, H. Cruz, Flávia.L. Ribeiro-Gomes, G.A. DosReis, A. N. Dutra, J. Clarêncio, C.I. de Oliveira, A. Barral, M. Barral-Netto, Cláudia. I. Brodskyn, Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with *Leishmania amazonensis*, *J. Leukoc. Biol.* 84 (2) (2008) 389–396, <https://doi.org/10.1189/jlb.0108018>.
- [70] I. Allays, I. Gyminova, C. Canet-Jourdan, P.E. Poubelle, L. Li, IL-32 γ Delays Spontaneous Apoptosis of Human Neutrophils through MCL-1, Regulated Primarily by the p38 MAPK Pathway, *PLoS ONE* 9 (10) (2014) e109256, <https://doi.org/10.1371/journal.pone.0109256>.
- [71] D. Kim, C.L. Haynes, The role of p38 MAPK in neutrophil functions: Single cell chemotaxis and surface marker expression, *Analyst.* 138 (2013) 6826–6833, <https://doi.org/10.1039/c3an01076g>.
- [72] Míriam.M. Chaves, D.C. Costa, Bárbara.F. de Oliveira, M.I. Rocha, José. A. Nogueira-Machado, Role PKA and p38 MAPK on ROS production in neutrophil age-related: Lack of IL-10 effect in older subjects, *Mech. Ageing Dev.* 130 (9) (2009) 588–591, <https://doi.org/10.1016/j.mad.2009.07.001>.