Impact of reactive oxygen species (ROS) on the control of parasite loads and inflammation in *Leishmania amazonensis* infection

Eric Henrique Roma1,3, Juan Pereira Macedo1, Grazielle Ribeiro Goes1, Juliana Lauar Gonçalves1,3, Waldionê de Castro1,4, Daniel Cisalpino2 and Leda Quercia Vieira1*

**Abstract**

**Background:** Reactive oxygen species (ROS) protect the host against a large number of pathogenic microorganisms. ROS have different effects on parasites of the genus *Leishmania*: some parasites are susceptible to their action, while others seem to be resistant. The role of ROS in *L. amazonensis* infection *in vivo* has not been addressed to date.

**Methods:** In this study, C57BL/6 wild-type mice (WT) and mice genetically deficient in ROS production by phagocytes (gp91phox−/−) were infected with metacyclic promastigotes of *L. amazonensis* to address the effect of ROS in parasite control. Inflammatory cytokines, parasite loads and myeloperoxidase (MPO) activity were evaluated. In parallel, *in vitro* infection of peritoneal macrophages was assessed to determine parasite killing, cytokine, NO and ROS production.

**Results:** *In vitro* results show induction of ROS production by infected peritoneal macrophages, but no effect in parasite killing. Also, ROS do not seem to be important to parasite killing *in vivo*, but they control lesion sizes at early stages of infection. IFN-γ, TNF-α and IL-10 production did not differ among mouse strains. Myeloperoxidase assay showed augmented neutrophils influx 6 h and 72 h post - infection in gp91phox−/− mice, indicating a larger inflammatory response in gp91phox−/− even at early time points. At later time points, neutrophil numbers in lesions correlated with lesion size: larger lesions in gp91phox−/− at earlier times of infection corresponded to larger neutrophil infiltrates, while larger lesions in WT mice at the later points of infection also displayed larger numbers of neutrophils.

**Conclusion:** ROS do not seem to be important in *L. amazonensis* killing, but they regulate the inflammatory response probably by controlling neutrophils numbers in lesions.

**Keywords:** *Leishmania amazonensis*, ROS, NOX2, Neutrophils, Inflammation

**Background**

Leishmaniasis is a spectrum of diseases caused by parasites of the genus *Leishmania*. This disease is endemic in 88 countries and affects two million people every year. The diseases may present themselves as cutaneous, mucocutaneous or visceral forms, depending on which species is involved in the infection [1].

In the well-established model of infection of mice with *L. major*, resistance to several parasite strains is mediated by the development of a Th1 immune response, while susceptibility is characterized by the development of a Th2 response [2–4], or to production of IL-10 [5]. However, C57BL/6 and C57BL/10 mice, which completely heal infection with *L. major*, develop chronic non-healing lesions when infected with *L. amazonensis* [6–8]. In addition, this susceptibility is independent of a Th2 response [6].

Macrophages are the main host cell for *Leishmania* spp. When infected with these parasites, macrophages sustain *Leishmania* spp. growth. However, when activated to produce nitric oxide, macrophages can kill *L. major* [9, 10]. Activation of macrophages is dependent on IFN-γ and TNF [9, 10]. Macrophages infected with *L. amazonensis*...
produce less TNF, even in the presence of IFN-γ [11, 12]. Hence, activation of L. amazonensis infected macrophages is deficient, at least in vitro [11, 12].

Reactive oxygen species derive from oxygen reduction, generating a group of highly reactive ions, molecules and radicals. ROS may be generated in mitochondria as respiratory chain products [13] and also participate in many biological processes, such as hormonal biosynthesis [14], cellular signalling [15] and destruction of intracellular pathogens [16]. ROS are also important effector agents against intracellular pathogens, induced by IFN-γ or Toll-like receptors [17, 18].

Phagocyte NADPH oxidases (NOX2) are a group of multimeric proteins composed by cytosolic chains (p67phox, p47phox and p40phox), a small G protein (rac1 or rac2) and membrane-associated subunits (gp91phox and p22phox) [19]. As other isoforms of NADPH oxidases, NOX2 catalyzes the production of superoxide anion (O2•−) by reducing oxygen, using NADPH as the electron donor [20]. The resulting superoxide may generate many reactive species including oxidized halogens, oxygen singlet and other free radicals. Phagocytic cells use these oxidants to kill intracellular pathogens, but these species can also cause tissue damage to host cells. Hence, NOX2 is strictly regulated and is activated upon specific stimuli, such as phagocytosis triggered by pathogen-associated molecular patterns (PAMPs) [21].

Gp91phox is essential for NOX2 function. It is responsible for molecular oxygen reduction by electrons provided by NADPH [22]. Humans or mice deficient in gp91phox present X-linked chronic granulomatous disease (CGD) [23, 24]. Patients with CGD have recurrent infections that can cause death as early as childhood. Although chemotaxis, degranulation and phagocytosis are normal, CGD patients show deficiency in destruction of phagocytosed microorganisms due to lack of metabolites generated from superoxide [25]. Accordingly, gp91phox knockout mice eventually develop CGD. These mice respond to chemically induced peritonitis with extensive neutrophil infiltration [23], and increased secretion of inflammatory cytokines and chemokines during lung infection by pneumococcal pneumonia [26].

The effect of ROS in in vivo infection caused by Leishmania spp. has been less well studied, since nitric oxide is believed to be the major effector molecule involved in parasite killing [27, 28]. In vitro studies show an irrelevant role of ROS in parasite killing by macrophages infected with L. major [29, 30] and L. guyanensis [31]. In vivo ROS control L. major parasitism in mice [30]. In L. donovani infection, ROS would be important only for short-term control of the parasites [32]. These differences in resistance to ROS observed during infection with different species of Leishmania make it necessary to investigate the role of ROS in other Leishmania spp.

A few papers have addressed the role of ROS during infection with L. amazonensis. The role of ROS has been addressed in vitro by some authors by measuring the amount of ROS produced by macrophages infected with L. amazonensis [11, 33] or with L. pifanoi, a parasite belonging to the Mexicana complex, like L. amazonensis [34]. ROS play a role in parasite killing of L. amazonensis by activated macrophages (with both IFN-γ and LPS) treated with ERK inhibitor [35]. However, the role of ROS produced upon phagocytosis of L. amazonensis on parasite killing and during in vivo infection has not yet been addressed.

Methods

Mice and ethics statements

C57BL/6 mice were obtained from the animal house of the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (CEBIO). Mice which genes for gp91phox subunit of NADPH oxidase were deleted by homologue recombination (B6.129S-Cybbtm1Din/J, here named gp91phox−/−) [23] were purchased from Jackson Farms (Glensville, NJ, USA). Animals were kept in conventional conditions with barriers, controlled light cycle and temperature. Food and water were provided ad libitum. All animals used in this study were 6 to 12 week-old. This project was approved by the local ethical committee under the protocol CETEA 031/09.

Parasites, infections and generation of Leishmania antigen

Leishmania amazonensis (IFLA/BR/67/PH8) was maintained in Grace’s medium as previously described [36]. Metacyclic promastigotes were purified in a ficoll gradient [37], washed, resuspended in phosphate buffered saline (PBS, pH 7.3) and counted. Inocula of 1 × 106 parasites/40 μl of PBS were injected in the mouse left hind footpad. Lesion development was followed by measuring the thickness of the footpad swelling using a digital micrometer (Starrett 727, Itu, SP, Brazil). Antigens were prepared from log phase promastigotes, washed in PBS and submitted to seven cycles of freezing in liquid nitrogen and thawing (37 °C). Suspensions were adjusted to a final concentration of 1 mg of protein/ml and kept at −70 °C until use. Protein concentration was assessed by the Lowry assay [38].

Quantification of parasites

Mice were sacrificed by cervical dislocation. The footpads were removed and disinfected in 70 % ethanol for 5 min and air-dried in the laminar flow hood. The footpads were cut in small parts and placed in RPMI medium (GIBCO, Grand Island, NY, USA) containing 100U/ml penicillin, 100 μg/ml streptomycin (GIBCO) and 125U/ml collagenase A (Sigma-Aldrich, Inc, St. Louis, MO, USA) for 2 h at 37 °C in a humidified
chamber and atmosphere containing 5% CO₂. After incubation the pieces of footpads were ground, filtered with a 40 μm cell strainer filter (BD Falcon, Franklin Lakes, NJ, USA) and washed with 10 ml of RPMI 0.05% DNase (Sigma-Aldrich). The homogenates were centrifuged at 50 × g for 4 min to remove large tissue debris and the supernatants were collected and centrifuged at 1,500 × g for 15 min. The sediment was re-suspended in 1 ml of complete RPMI (GIBCO) (RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), 100U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (GIBCO BRL). Fifty microliters of the suspension were serially diluted in a 96-well plate containing 150 μl of Grace’s insect medium supplemented with 20% heat-inactivated FBS, 100U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (GIBCO) (Grace’s complete medium) in each well. The samples were serially diluted in Grace’s complete medium (1:4) in triplicates. Pipette tips were discarded after each dilution. Plates were cultured for 10 days in BOD incubator at 25°C and the last positive dilution was registered as the titre. Results are expressed as the negative logarithm of the titre.

Luminometry assay
Mice were injected intraperitoneally with 2 ml of 4% thioglycollate (BD Biosciences, Franklin Lakes, NJ, USA). After 3 days, mice were euthanized and the peritoneum cells were harvested by repeated cycles of aspiration and re-injection with 10 ml of cold PBS in a 10 ml syringe with a 24G needle. Cells were centrifuged at 4°C, 1,500 × g for 10 min, counted in a hemocytometer and the concentration was adjusted to 1 × 10⁶ cells/100 μL of complete RPMI without phenol red. Cells (1 × 10⁶ cells/well) were plated in 96 well opaque plates (NUNC, Rochester, NY, USA) together with 0.05 mM of luminol (5-amino-2,3-dihydro-1,4-phthalalizinedione, Sigma-Aldrich). Immediately before the measurement, L. amazonensis metacyclic promastigotes were added in the proportion of 5 parasites per macrophage. The measurement was followed for 90 min with one minute of interval between the measurements. The production of ROS was assessed by the light intensity generated by the reaction between ROS and luminol and expressed as relative light units.

In vitro assays for parasite burden
Macrophages were isolated from the peritoneal cavity of mice 3 days after injection of 2 ml of 4% thioglycollate medium (BD Biosciences, Franklin Lakes, NJ, USA) into the peritoneal cavity. After this time, mice were euthanized and the peritoneum cells were harvested by repeated cycles of aspiration and re-injection with 10 ml of cold PBS in a 10 ml syringe with a 24G needle. More than 80% of the cells harvested were macrophages. The cells were centrifuged at 4°C, 1,500 × g for 10 min and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), 1% penicillin-streptomycin and 2 mM L-glutamine. Macrophages were counted in a hemocytometer prior to seeding 5 × 10⁶ cells into each well of a 24-well plate and incubated at 37°C, 5% CO₂ for 2 h. After this time, L. amazonensis metacyclic promastigotes were added in the proportion of 5 parasites per macrophage during 4 h. After this period, cells were washed three times with phosphate-buffered saline (PBS, pH 7.3) to remove extracellular parasites. Cells were fixed or reincubated with medium for 72 h before fixation with methanol. Coverslips with attached macrophages were stained with Panotico (Laborclin, Pinhais, PR, Brazil) and a minimum of 200 macrophages per coverslip were counted. The results were expressed as an infection index (% infected macrophages x number of amastigotes/total number of macrophages). The following drugs were used in these assays: apocynin (APO) (300 μM; Sigma-Aldrich) and N-acetyl-cysteine (NAC) (1 mM; Sigma-Aldrich) and H₂O₂ (100 μM). Drugs were added to the cells 2 h before and immediately after infection.

Real time PCR
Total RNA, obtained from lesions at 4, 8, 12 and 16 weeks post-infection, was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. One μg of total RNA obtained from the lesions or lymph nodes was reverse transcribed using reverse transcriptase (Promega, Southampton, UK) and oligo (dT) 15-mer primer (Promega, Southampton, UK). PCR amplification was performed with a programmable thermal cycler (Perkin-Elmer 2400, Waltham, MA, USA). The cDNA amplification protocol was as follows: 2 min at 50°C, activation of AmpliTaq at 95°C for 10 min, melting at 95°C for 15 s. For the annealing and final extension, the samples were heated at 60°C for 1 min for 45 cycles. For dissociation curve, the samples were heated at 95°C for 15 s, following cooling at 60°C for 5 s. Finally, the samples were cooled for 1 min at 4°C.

The amplification of cDNA was made using specific primers as follow: IFN-γ (forward TCAAGTGGCATA-GATGTGGAAGAA, reverse TGGCTCTGCGAGGATTT TCATG), IL-4 (forward ACAGGAGAAGGGACCCCA, reverse GAAGCCCTACAGACGAGCTCA), IL-10 (forward GTGGCAATATCCGGA, reverse ACCCTGCT CCACTGCTTCTG), TNF-α (forward TTCTGTCT ACTGAACTTCGGGGTGATCGGTCC, reverse GTAT GAGATAGCAAATCGGCTGACGGTGTGGG), IL-1β (forward CAACCAACAGTTGATATTCTCCAT, reverse GATCCACACTTCCAGCTGCA), iNOS (forward CCC TTCCGAGTTTCTGGACGAC, reverse GGCTGT
CAGAGCCTCGTGCTTTTGG). The reactions were developed in the ABI PRISM®7900HT (Applied Biosystems, Foster City, CA, USA) using 20 % of reaction in cDNA volume and 15 µl of total PCR mixture. All reactions were performed in duplicate using SYBR Green Master Mix (Applied Biosystems) according to manufacturer’s instructions. Finally, the samples were cooled for 1 min at 4 °C. The specific cDNAs were normalized according to the expression of ribosomal 18 s gene (forward TACCACATC CAAGAAGGCA, reverse TGCCCTCCAATGGGC CTC) based in ΔCT calculation. The results were expressed as fold increase of target gene expression compared to ribosomal 18 s gene expression.

Cytokine and nitrite assays

Single cell suspensions of draining lymph nodes (dLNs) from mice infected for 8, 12 and 16 weeks post-infection plated in 24 well plates at 5 × 10⁶ cells/mL (Nunclon, Nunc) and stimulated with L. amazonensis antigen (50 µg/ml) for 72 h at 37 °C and 5 % CO₂. Concanavalin A was used as positive control (10 µg/ml) of cytokine production. The levels of IFN-γ, IL-10, IL-6, IL-17a were measured in supernatant using appropriate kits (BD OptEIA™, BD Biosciences, Franklin Lakes, NJ, USA) following manufacturer’s instructions. Detection levels were 12.5 pg/ml for all cytokines measured.

Nitrites were measured in supernatants of peritoneal macrophage cultures infected with L. amazonensis. Briefly, peritoneal macrophages were harvested as described above. The cells (5 × 10⁶) were plated in 48 well plates in 500 µl of complete RPMI. After 16 h, the cultures were washed to remove the non-adherent cells, and cells were activated with 50U/ml of IFN-γ (BD biosciences, Franklin Lakes, NJ, USA) and 100 ng/ml of LPS (Sigma-Aldrich) for 4 h at 37 °C and 5 % CO₂. Following the activation, the cells were infected with L. amazonensis metacyclic promastigotes at 10 parasites per macrophage. After 4 h of infection, the cells were washed, re-stimulated with IFN-γ and LPS at the same concentrations used before and incubated for 48 h at 37 °C and 5 % CO₂. The supernatants of in vitro infected macrophages were collected 48 h after infection and the levels of IL-6, TNF-α, IL-17A and CXCL-1, MCP-1 and IL-10 were measured using appropriate kits (BD OptEIA™, BD Biosciences, Franklin Lakes, NJ, USA) following manufacturer’s instructions.

After 48 h, 50 µl of supernatants were collected and used to quantify nitrite using the Griess method [39] in 96 well plates. After 10 min of reaction the plates were read in a plate reader (EZ read 400, Biochrom, Cambridge, UK) at 540 nm.

Myeloperoxidase assay

Neutrophil accumulation in the infected footpads was measured by assaying myeloperoxidase (MPO) activity.

Briefly, the footpads were infected as described in item 2.2. Six and 72 h post-infection, mice were euthanized, the footpads were removed and snap-frozen in liquid nitrogen. On thawing, the tissue (100 mg of tissue per 1.9 ml of buffer) was homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M Na₂PO₄, 0.015 M sodium-ethylendiaminetetraacetic acid), centrifuged at 260 × g for 10 min and the pellet subjected to hypotonic lyses (15 ml of 0.2 % NaCl solution followed by 30 s of equal volume of a solution containing 1.6 % NaCl and 5 % glucose). After further centrifugation, the pellet was resuspended in 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5 % hexadecyltrimethylammonium bromide and re-homogenized. One ml aliquots of the suspension were transferred into 1.5 ml conical microtubes followed by three freeze-thaw cycles using liquid nitrogen. The aliquots were then centrifuged for 15 min at 10,000 × g, the pellet was re-suspended to 1 ml. Myeloperoxidase activity in the re-suspended pellet was assayed by measuring the change in optical density (OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H₂O₂ (0.5 mM). Results were expressed as OD units.

Statistical analysis

Statistical significance between groups was determined by the unpaired, two-tailed Student’s t test using Prism software (GraphPad, La Jolla, CA, USA). P values < 0.05 were considered significant.

Results

Leishmania amazonensis metacyclic promastigotes are capable of inducing a respiratory burst in mouse macrophages

ROS are very important for the elimination of a variety of intracellular pathogens. Hence, we first analysed if L. amazonensis metacyclic promastigotes triggered the respiratory burst in murine macrophages. We followed ROS production for 90 min using luminometry [40]. Macrophages produced ROS within two minutes of infection, maximum production was found between 15 and 30 min after addition of parasites (Fig. 1). Once it was determined that L. amazonensis metacyclic promastigotes could stimulate ROS production by macrophages, we proceeded to investigate if ROS would have an effect on the course of infection with this parasite.

Gp91phox−/− mice develop larger lesions after infection with L. amazonensis

To address the importance of ROS during L. amazonensis infection, we infected mice deficient in the gp91phox subunit of NOX2 (gp91phox−/−). This subunit is responsible for transferring electrons from NADPH to oxygen, generating superoxide anion. Consequently, gp91phox−/− mice cannot produce ROS through NOX2 [23].
We observed larger lesions in gp91phox−/− mice from five to seven weeks post-infection with L. amazonensis (Fig. 2a), when compared to lesions in WT mice. However, 11 weeks post-infection, lesions in gp91phox−/− started to decline and were significantly smaller than lesions in WT mice until week 14 of infection. Lesions in both groups declined and were similar at weeks 15 and 16 weeks post-infection.

Regardless of differences in lesion sizes observed between gp91phox−/− and WT mice, we found no differences in parasite loads, neither in lesions nor in draining lymph nodes, at 4, 8, 12 and 16 weeks post-infection (Fig. 2b, c). In addition, we found no differences between in vitro infection of WT and gp91phox−/− macrophages (Fig. 2d). Addition of apocynin, an inhibitor of NOX2, or N-acetyl-cysteine to WT macrophages confirmed that L. amazonensis is not susceptible to oxidative stress within macrophages (Fig. 2e).

Cytokine production in footpads, draining lymph nodes and in vitro-infected macrophages

Since IFN-γ and TNF-α are crucial to eliminate Leishmania in macrophages, we investigated the mRNA expression of these cytokines in gp91phox−/− mice. No differences in mRNA levels for IFN-γ or TNF-α in footpads were detected in any of the times measured (Fig. 3a, b). However, we found higher expression of IL-1β mRNA in WT mice at 12 and 16 weeks of infection (Fig. 3e). Moreover, mRNA levels of IL-4 were higher in gp91phox−/− at the last time point measured (Fig. 3c). No differences in IL-10 mRNA levels between groups were observed (Fig. 3d).

We did not observe differences in IFN-γ production by draining lymph node (dLN) cells 8, 12 and 16 weeks post-infection (Fig. 4a). Larger production of IL-10 was found in gp91phox−/− mice 16 weeks after infection (Fig. 4b).

Draining lymph node cells from gp91phox−/− mice produced more IL-17A 8 weeks post-infection than cells from WT mice. IL-17 production by dLN cells dropped at 12 and 16 weeks post-infection, and no differences were found between the groups (Fig. 4c). No differences in IL-17 mRNA were found in lesions (data not shown). Interestingly, despite the differences found in IL-1β mRNA expression levels 12 and 16 weeks post-infection in WT infected footpads (Fig. 3e), we could not observe augmented levels of this cytokine in dLNs of WT in all periods measured when compared to gp91phox−/− dLNs (Fig. 4d).

We also determined the production of cytokines by in vitro-infected macrophages (Fig. 5). We found that macrophages from gp91phox−/− and WT mice produced similar levels of IL-6, TNF-α, IL-17A and CXCL-1, MCP-1 and IL-10, regardless of infection with L. amazonensis. However, upon stimulation with LPS (a stimulus for TNF-α production) and IFN-γ, infected macrophages from gp91phox−/− mice produced higher levels of IL-6, TNF-α (albeit not consistently in all experiments), IL-17A and IL-10. CXCL-1 production was similar in both macrophages, while MCP-1 was higher in WT macrophages than in gp91phox−/− cells.

Deficiency in ROS production is not compensated by increase in iNOS expression

ROS and RNS may play complementary roles in killing intracellular parasites [32, 41]. However, in our experiments, gp91phox−/− mice harboured parasites similarly to WT. This could be due to an irrelevant role of ROS in infection with L. amazonensis or to a compensatory over-production of nitric oxide. We investigated the mRNA expression of inducible nitric oxide synthase (iNOS) in lesions and the production of nitric oxide (NO) by peritoneal macrophages in gp91phox−/− mice. Messenger-RNA levels of nitric oxide in lesions were similar between groups at all times measured. Both groups displayed higher expression of iNOS at 8 weeks post-infection, and lower levels of expression 4, 12 and 16 weeks post-infection (Fig. 6a). Thus, ROS deficiency did not increase the transcription of iNOS genes. In addition, in vitro-infected IFN-γ-activated macrophages from gp91phox−/− and WT mice produced similar levels of NO (Fig. 6b).

ROS influence the migration of neutrophils after infection with L. amazonensis

Neutrophils are important to eliminate invading microorganisms, and one of the mechanisms of this elimination is...
by ROS production. Hence, we investigated neutrophil migration to the site of infection in gp91phox−/− and WT mice. First, we infected mice with 1 × 10⁶ metacyclic promastigote forms of L. amazonensis in the right hind footpad and followed until 16 weeks. a Footpad thickness, **p < 0.01 between WT and gp91phox−/− mice. b Parasite loads in footpads of infected mice 4, 8, 12 and 16 weeks post-infection. c Parasite loads found in draining lymph nodes of infected mice 4, 8, 12 and 16 weeks post-infection. Data are shown as mean ± SD from one representative experiment of three, n = 5 for each experiment. d and e Inflammatory macrophages obtained from peritoneal cavity of C57BL/6 and gp91phox−/− mice were infected with L. amazonensis metacyclic promastigotes. The cells were washed to remove extracellular parasites and either fixed or re-incubated with medium for 72 h. The slides were stained and counted to determine the infection index. A minimum of 200 macrophages were counted per group in triplicate. d Infection index for C57BL/6 and gp91phox−/− macrophages four and 72 h post in vitro infection. e Infection index in macrophages from WT pre-incubated with N-acetyl-cysteine (NAC) or apocynin (APO) or from gp91phox−/− mice 72 h post in vitro infection.
infection. More interestingly, MPO activity persisted 72 h after infection (Fig. 7a). We also followed the accumulation of granulocytes throughout the course of infection by flow cytometry, using the Ly6G marker. At eight weeks post-infection there were more neutrophils in footpads from gp91$^{phox−/−}$ mice (Fig. 7b, c). On the other hand, there were more neutrophils at 12 weeks post-infection in WT mice.

**Discussion**

In this work, we analysed the importance of ROS production by the host on the outcome of infection caused by *L. amazonensis*. C57BL/6 mice are unable to spontaneously heal lesions after subcutaneous infection with *L. amazonensis*, but develop chronic lesions that do not progress indefinitely [6, 8]. We observed increase in paw swelling of infected WT mice until week 7 post-infection, followed by a slight decrease and lesion stabilization after this time point. Indeed, our results are supported by other works showing similar lesion kinetics during *L. amazonensis* infection in C57BL6 mice [42–44]. A role for IFN-$γ$ and iNOS in this partial resistance to the parasite is clear, especially at later times of infection, since *L. amazonensis*-infected IFN-$γ^{−/−}$ and iNOS$^{−/−}$ mice develop larger non-healing lesions than wild type mice [44, 45]. ROS have been shown to play a role in the containment of metastasis to spleen and lymph nodes in experimental *L. major* infection [30]. Macrophages [29, 30] and neutrophils [30] respond to infection with *L. major* with ROS production, as shown in vitro. However, killing of *L. major* by IFN-$γ$-activated macrophages is dependent on NO production, but not on the production of superoxide or peroxynitrite [29], hampering the role of ROS in resistance to this parasite. *L. guyanensis* amastigotes, on
the other hand, die inside BALB/c macrophages through an apoptosis-like process mediated by parasite-induced ROS [31]. However, little is known about the role of ROS in resistance to *L. amazonensis*. It has been reported that *L. amazonensis* triggers less ROS production by macrophages from CBA mice in vitro 30 min after infection [33]. However, production of ROS by activated macrophages from C57BL/6 mice infected with *L. amazonensis* 30 min and 5 days post-infection has been reported [46]. Production of ROS was dependent on gp91<sub>phox</sub> and ROS mediated parasite killing in vitro. In addition, *in vivo* ROS production by C57BL/6 mice during *L. amazonensis* infection has been reported [47, 48]. The production of ROS was determined indirectly by detection of nitrotyrosine, which provides evidence of peroxynitrite production. Peroxynitrite is produced by the reaction of ROS (produced by NADPH oxidase) and NO (produced by iNOS), these enzymes co-localize in the phagolysosome, speaking for their role in the production of peroxynitrite and damage to the parasite [47].

In our experiments, using metacyclic promastigote forms of PH8 *L. amazonensis* strain and thioglycollate-elicited macrophages from CBA mice, *L. amazonensis* induces ROS production by peritoneal macrophages *in vitro* (Fig. 1). Parasites of the genus *Leishmania* possess several mechanisms to escape ROS in phagolysosomes. *L. pifanoi* is capable of blocking the maturation of the gp91<sub>phox</sub> subunit of RAW 264.7 cells, avoiding the NADPH oxidase complex formation [34]. *L. donovani* LPG blocks the translocation of p47<sub>phox</sub> and p67<sub>phox</sub> to the phagosome, inhibiting NADPH oxidase activation [49]. *L. amazonensis* promastigotes developed strategies to resist to ROS; however, the mechanisms of this resistance have not been determined yet. It is possible that *L. amazonensis* does not inhibit NOX2, since *L. amazonensis*-infected CBA macrophages respond to infection with *L. major* similarly to uninfected macrophages [33]. On the other hand, the host produces ROS in response to a variety of stimuli, such as activated complement [50], binding to IgG Fc receptor (FcγR) [51] and Toll-like receptor 2 [17], during *Leishmania* spp. infection. Considering the sophisticated mechanisms used by the parasites to escape damage by ROS and the variety of mechanisms by which the host achieves ROS production, allied with the susceptibility of some species of *Leishmania* to ROS [52], it was reasonable to infer that ROS may play a role in resistance to *L. amazonensis*. Lesion development in gp91<sub>phox</sub>−/− and WT mice after infection with *L. amazonensis* was quite different. In the first seven weeks of infection, gp91<sub>phox</sub>−/− mice presented larger lesions, but the same number of parasites as WT mice. Coherently, macrophages from WT and gp91<sub>phox</sub>−/− mice harboured similar numbers of parasites and produced similar levels of NO when activated with IFN-γ, and similar levels of message for iNOS were found in footpads from both infected mouse strains. In addition, cytokine levels at the site of infection, as well as IFN-γ, IL-1β and IL-10 production by antigen-stimulated lymph node cells were
similar among groups. However, IL-17 production by draining lymph node cells was higher in gp91phox−/− mice than in WT at 8 weeks of infection. Interestingly, MPO activity was higher in gp91phox−/− footpads than in WT at 6 and 72 h post-infection. In addition, more Ly6G+ cells were found at the site of infection 8 weeks post-infection in gp91phox−/− mice, subsequently to when gp91phox−/− mice displayed larger lesions than WT mice. On the other hand, at 12 weeks of infection, when WT mice had larger lesions, they also had more neutrophils at the site of infection. These data suggest that ROS could have a relevant role in modulating the inflammatory infiltrate, but irrelevant action

**Fig. 6** INOS mRNA expression in footpads and nitrite production by infected thioglycollate-elicited macrophages. **a** Wild type (WT) and gp91phox−/− mice were infected with 1 × 10⁶ metacyclic promastigote forms of *L. amazonensis* in the right hind footpad and followed for 16 weeks. iNOS mRNA levels were normalized by 18S mRNA expression 4, 8, 12 and 16 weeks post-infection. **b** Thioglycollate-elicited macrophages were harvested from the peritoneal cavity of WT or gp91phox−/− mice 3 days after stimulation. Macrophages were infected with *L. amazonensis* metacyclic promastigotes (10 parasites per macrophage) for 4 h and cultures were washed and re-stimulated or not with IFN-γ plus LPS at the same concentrations used before. After 48 h of infection supernatants were collected and used to measure nitrite levels by Griess reaction. Data are shown as mean ± SD of one representative experiment of four, n = 5 for each experiment.
in parasite killing in vivo. The reason for the larger lesions up to 7 weeks (maybe coinciding with larger numbers of neutrophils) and smaller lesions at later time points (also coinciding with smaller numbers of neutrophils) is not yet known. Ablation of neutrophils early in infection with L. amazonensis, in our experiments, caused no effect in the outcome of infection in C57BL6 mice [53]. However, our results here suggest that the persistence of this otherwise short-lived cell may exacerbate pathology. Larger neutrophil infiltrates are found in NOX2-deficient mice [23], and that would explain, possibly, the larger lesions at the earlier time points of infection. As stated above, at the later time points a larger neutrophil infiltrate was found in WT mice. The reason for the variation in these numbers in the two mice is unknown at this point. We could, however, speculate that the neutrophil infiltrate is more precocious in gp91phox−/− mice and more precociously resolved than in WT mice. Eventually, lesions were the same in both mouse strains.

Humans deficient on functional NADPH oxidase develop chronic granulomatous disease (CGD), which is characterized by recurrent infections and limitations in elimination of intracellular microorganisms; gp91phox−/− mice kept in less sanitary conditions may also develop CGD [23, 25]. Moreover, gp91phox−/− mice show high numbers of neutrophils during peritonitis caused by chemical agents [23] as well as increased inflammatory cytokine and chemokine production [26]. There are solid evidences implicating ROS

**Fig. 7** Myeloperoxidase (MPO) activity and Ly6G+ cells at the infection site in wild type (WT) and gp91phox−/− mice. Mice were infected with 1 × 10^6 metacyclic promastigote forms of L. amazonensis (PH8) in the right hind footpad, or injected with PBS. a After 6 h or 72 h of infection, footpads were removed and used to assay MPO activity to estimate neutrophil numbers in the acute phase of infection. b Flow cytometry of Ly6G+ cells in footpads 4, 8, 12 and 16 weeks post-infection, performed as described in the Methods section. Data are shown as mean ± SD from one representative experiment of two, n = 5 for each experiment, *P < 0.05, **P < 0.01 between WT and gp91phox−/− mice. c Representative dot plots of Ly6G+ cells recovered from infected footpads after 4, 8, 12 and 16 weeks of infection. The dot plots represent percentage of Ly6G+ cells gated on side scatter versus forward scatter cell properties.
in the induction of neutrophil apoptosis [54–60]. Consequently, it is possible to infer that a decrease in neutrophil apoptosis culminates with the hyper-inflammation seen in CGD [56, 61–64]. Accordingly, at early time points (6 and 72 h, Fig. 7a) we observed higher numbers of neutrophils at the infection site in gp91phox−/− mice. This fact could be related with the impaired capacity of neutrophils from gp91phox−/− mice to start the apoptotic programme via ROS. Moreover, other mechanisms such as persistent cellular activation [65], attenuation of ROS dependent Ca2+ signaling [66, 67] and oxidation of transcriptional factors and phosphatases via ROS [68] could contribute to the larger lesions in gp91phox−/− mice. Despite extensive data in the literature reporting increased expression of inflammatory cytokines in gp91phox−/− mice [66, 67, 69, 70], we could not detect alterations in cytokine mRNA levels (Fig. 3a, b) in lesions. Nevertheless, macrophages infected in vitro with L. amazonensis and activated with IFN-γ produced higher levels of IL-6, TNF-α, IL-17A and IL-10 (perhaps in response to high levels of TNF-α [71]) and lower levels of MCP-1 when compared to cytokine production by WT macrophages. In addition, a larger production of IL-17A by lymph node cells was found at 8 weeks of infection, which correlated with the larger neutrophil infiltrate. Th17 response is related to increased neutrophil migration in many experimental models [72–75]. The higher production of IL-17A observed herein could contribute to the migration of neutrophils to lesions and promote a larger inflammatory infiltrate in gp91phox−/− mice. Surprisingly, after 11 weeks of infection, lesions in gp91phox−/− mice started to decrease and became smaller that lesions in the WT group. Again, no differences in parasite numbers were found between groups. However, the mRNA levels IL-1β were higher in WT mice at later time points, indicating an inflammatory status. Indeed, IL-1β expression could be dependent of ROS release [76], which could explain the decrease of footpad swelling and the low concentration mRNA levels of IL-1β observed in gp91phox−/− mice at this time point. Gp91phox−/− footpads expressed higher mRNA levels for IL-4, which could indicate an anti-inflammatory status at later times of infection. No differences in IL-10 levels were found throughout infection.

**Conclusion**

To the best of our knowledge, this is the first report addressing the role of NOX2 in experimental murine infection with *L. amazonensis*. Our results indicate that ROS might regulate the inflammation caused in *L. amazonensis* infection, but does not affect parasite control. The mechanisms used by *L. amazonensis* to avoid killing by ROS still need to be addressed.

**Authors’ contributions**

All authors read and approved the final version of the manuscript. EHR, JPM and LQV conceived and designed the experiments. EHR, JPM, GRG, ILG, WC and DC performed the experiments. EHR, JPM, GRG and LQV analysed the data. EHR, JPM, GRG, WC and LQV contributed to the writing of the manuscript.

**Acknowledgements**

EHR, GRG, ILG, DC and LQV are CNPq fellows. JPM was a CAPES fellow. This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, CDS-RED-00315-14; APQ-02308-13 and APQ-01419-14). The authors are members of the INCT de Processos Redox e Biomediçao-Redoxoxma (FAPESP/CNPq/CAPES, proc: 573530/2008-4). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Author details**

1 Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. 2 Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. 3 Current address: Instituto Nacional de Infectologia, Fiocruz, Rio de Janeiro, RJ, Brazil. 4 Current address: Laboratory of Malaria and Vector Research, Vector Molecular Biology Section, National Institutes of Health, NIAID, Rockville, MD, USA.

Received: 5 November 2015 Accepted: 23 March 2016

**Published online**: 07 April 2016

**References**

1. Pace D. Leishmaniasis. J Infect. 2014;69 Suppl 1:S10–8.
2. Sadick MD, Locksley RM, Tubbs C, Raif HV. Murine cutaneous leishmaniasis: resistance correlates with the capacity to generate interferon-gamma in response to Leishmania antigens in vitro. J Immunol. 1986;136(5):655–61.
3. Locksley RM, Heizel FL, Sadick MD, Holiday RJ, Gardner JD. Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets. Ann Inst Pasteur Immunol. 1987;138(5):744–9.
4. Sadick MD, Heizel FF, Shigekane VM, Fisher WL, Locksley RM. Cellular and humoral immunity to Leishmania major in genetically susceptible mice after in vivo deletion of L3T4+ T-cells. J Immunol. 1987;139(4):1303–9.
5. Anderson CF, Mendez S, Sacks DL. Nonhealing infection despite Th1 polarization produced by a strain of Leishmania major in C57BL/6 mice. J Immunol. 2005;174(3):2934–41.
6. Afonso LC, Scott P. Immune responses associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. Infect Immun. 1993;61(7):2952–9.
7. Jones DE, Ackermann MR, Wille U, Hunter CA, Scott P. Early enhanced Th1 response after Leishmania amazonensis infection of C57BL/10 intraperitoneal-10 deficient mice does not lead to resolution of infection. Infect Immun. 2002;70(4):2151–8.
8. Soong L, Chang CH, Sun J, Longley Jr BJ, Ruddle NH, Flavell RA, McMahon-Pratt D. Role of CD4+ T cells in pathogenesis associated with *Leishmania amazonensis* infection. J Immunol. 1997;158(11):5374–83.
9. Green SJ, Crawford RM, Hockmeyer JT, Wietzerbin J, White LA, Nacy CA. Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of necrosis factor-alfa. J Immunol. 1990;145(2):4290–7.
10. Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S. Macrophage killing of *Leishmania* parasite in vivo is mediated by nitric oxide from L-arginine. J Immunol. 1990;144(2):4794–7.
11. Gomes IN, Galabrich AF, Tavares Rda S, Wietzerbin J. Differential properties of CBA/J mononuclear phagocytes recovered from an experimental infection site and probed with two different species of *Leishmania*. Microbes Infect. 2003;5(4):251–60.
12. Horta MF, Mendes BP, Roma EH, Noronha FS, Macedo JP, Oliveira LS, Duarte MM, Vieira LQ. Reactive oxygen species and nitric oxide in cutaneous leishmaniasis. J Parasitol Res. 2012;2012:203818.
13. Rada B, Leito TL. Oxidative innate immune defenses by Nos/Duxo family NADPH oxidases. Contrib Microbiol. 2008;15:164–87.
14. Chan YC, Leung P. The Renin-angiotensin system and reactive oxygen species: implications in pancreatitis. Antioxid Redox Signal. 2011;15(10):2743–55.
amastigotes avoid... in vitro Trypanosoma cruzi:...

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis...

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.

amastigotes by an L-arginine-dependent... revealed by... Leishmania amazonensis: J Microbiol Immunol Infect. 2014;49(2):271-5.
60. Coxon A, Rieu P, Barkelow FJ, Askari S, Sharpe AH, von Andrian UH, Arnaout MA, Mayadas TN. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. Immunity. 1996;5(6):653–66.

61. van de Loo FA, Bennink MB, Arntz OJ, Smeets R, Lubberts E, Joosten LA, Lent PL, Coenen-de Roo CJ, Cuzzocrea S, Segal BH, et al. Deficiency of NADPH oxidase components p47phox and gp91phox caused granulomatous synovitis and increased connective tissue destruction in experimental arthritis models. Am J Pathol. 2003;163(4):1525–37.

62. Brown JR, Goldblatt D, Buddle J, Morton L, Thrasher AJ. Diminished production of anti-inflammatory mediators during neutrophil apoptosis and macrophage phagocytosis in chronic granulomatous disease (CGD). J Leukoc Biol. 2003;73(5):591–9.

63. Hampton MB, Fadel B, Orrenius S. Redox regulation of the caspases during apoptosis. Ann N Y Acad Sci. 1998;854:328–35.

64. Hampton MB, Vissers MC, Keenan JJ, Winterbourn CC. Oxidant-mediated phosphatidylserine exposure and macrophage uptake of activated neutrophils: possible impairment in chronic granulomatous disease. J Leukoc Biol. 2002;71(3):775–81.

65. Schappi M, Deffert C, Fiette L, Gavazzi G, Herrmann F, Bello D, Krause KH. Branched fungal beta-glucan causes hyperinflammation and necrosis in phagocyte NADPH oxidase-deficient mice. J Pathol. 2008;214(4):434–44.

66. Geiszt M, Kapus A, Nemet K, Farkas L, Ligeti E. Regulation of capacitative Ca2+ influx in human neutrophil granulocytes. Alterations in chronic granulomatous disease. J Biol Chem. 1997;272(42):26471–8.

67. Rada BK, Geiszt M, Van Bruggen R, Nemet K, Roos D, Ligeti E. Calcium signalling is altered in myeloid cells with a deficiency in NADPH oxidase activity. Clin Exp Immunol. 2003;132(1):53–60.

68. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007;87(1):245–313.

69. Hatanaka E, Carvalho BT, Condino-Neto A, Campa A. Hyperresponsiveness of neutrophils from gp 91phox deficient patients to lipopolysaccharide and serum amyloid A. ImmunoLett. 2004;94(1–2):43–6.

70. Lekstrom-Himes JA, Kuhns DB, Alvord WG, Gallin JJ. Inhibition of human neutrophil IL-8 production by hydrogen peroxide and dysregulation in chronic granulomatous disease. J Immunol. 2005;174(1):411–7.

71. Barsig J, Kusters S, Vogt K, Volk HD, Tiets G, Wendel A. Lipopolysaccharide-induced interleukin-10 in mice: role of endogenous tumor necrosis factor-alpha. Eur. J Immunol. 1995;25(10):2888–93.

72. Hoshino H, Laan M, Sjostrand M, Lottvall J, Skoogh BE, Linden A. Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. J Allergy Clin Immunol. 2000;105(1 Pt 1):143–9.

73. Wittowski J, Pavlaczcyk K, Breborowicz A, Schreun A, Kuzan-Pawlaczcyk M, Wisniewska J, Polubinska A, Fries H, Gahl GM, Frei U, et al. IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. J Immunol. 2000;165(10):5814–21.

74. Kelly MN, Kolls J, Happel K, Schwartzman JD, Schwarzenberger P, Combe C, Moretto M, Khan IA. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against Toxoplasma gondii infection. Infect Immun. 2005;73(1):617–21.

75. Yu JJ, Ruddy MJ, Wong GC, Sfintescu C, Baker PJ, Smith JB, Evans RT, Gaffen SL. An essential role for IL-17 in preventing pathogen-initiated bone destruction: recruitment of neutrophils to inflamed bone requires IL-17 receptor-dependent signals. Blood. 2007;109(9):3794–802.

76. Bonizzi G, Pilette J, Merville MP, Bours V. Cell type-specific role for reactive oxygen species in nuclear factor-kappaB activation by interleukin-1. Biochem Pharmacol. 2000;59:7–11.