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Annexin A1 improves immune responses and control of tissue parasitism during *Leishmania amazonensis* infection in BALB/c mice

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ABSTRACT

Leishmaniases, a group of diseases caused by the species of the protozoan parasite *Leishmania*, remains a significant public health concern worldwide. Host immune responses play a crucial role in the outcome of *Leishmania* infections, and several mediators that regulate inflammatory responses are potential targets for therapeutic approaches. Annexin A1 (AnxA1), an endogenous protein endowed with anti-inflammatory and pro-resolving properties, has emerged as a potential player. We have shown that during *L. braziliensis* infection, deficiency of AnxA1 exacerbates inflammatory responses but does not affect parasite burden. Here, we have investigated the role of AnxA1 in *L. amazonensis* infection, given the non-healing and progressive lesions characteristic of this infectious model. Infection of AnxA1 KO BALB/c mice resulted in increased lesion size and tissue damage associated with higher parasite burdens and enhanced inflammatory response. Notably, therapeutic application of the AnxA1 peptidomimetic Ac2–26 improves control of parasite replication and increases IL-10 production *in vivo* and *in vitro*, in both WT and AnxA1 KO mice. Conversely, administration of WRW4, an inhibitor of FPR2/3, resulted in larger lesions and decreased production of IL-10, suggesting that the effects of AnxA1 during *L. amazonensis* infection are associated with the engagement of these receptors. Our study illuminates the role of AnxA1 in *L. amazonensis* infection, demonstrating its impact on the susceptibility phenotype of BALB/c mice. Furthermore, our results indicate that targeting the AnxA1 pathway by using the Ac2–26 peptide could represent a promising alternative for new treatments for leishmaniasis.

1. Introduction

The term Leishmaniases designates a set of diseases caused by the protozoa genus *Leishmania*, including visceral leishmaniasis (VL) and tegumentary leishmaniasis (TL) as the main clinical forms [1]. In Latin American countries, two species are responsible for more than 70% of cases of TL, *Leishmania (Viannia) braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* [2]. Different clinical manifestations of TL are associated with *Leishmania amazonensis* infection, such as localized cutaneous Leishmaniasis (LCL), diffuse cutaneous Leishmaniasis (LCD) and even VL. *L. amazonensis* has been also isolated from dogs with VL, strengthening the importance of *L. amazonensis* as an etiologic agent of Leishmaniases in the New World [3–5].

Successful control of *Leishmania* infections involves the coordination of different innate and adaptive immune mechanisms of the host. *L. amazonensis* parasites are highly skilled in subverting immune responses, such as macrophage activation in order to survive and persist in host cells [6,7]. Acute inflammation is a necessary process for protection of host against exogenous and endogenous factors that ultimately needs to be tightly regulated to ensure minimal tissue damage. After activation of an inflammatory process, several anti-inflammatory/pro-resolving mediators act to return tissue to homeostasis [8]. One of these mediators, Annexin A1 (AnxA1), is a protein endowed with several anti-inflammatory and pro-resolving effects, including decreasing the

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production of inflammatory mediators, modulating leukocyte infiltration and activation, enhancing microbial clearance and promoting tissue healing [9,10]. Anti-inflammatory and pro-resolving actions of AnxA1 and of its mimetic peptide, Ac2–26, are thought to occur through the FPR2/ALX, a G protein-coupled receptor belonging to the formyl peptide receptor (FPR) family [11–14].

Modulation of AnxA1 expression during *Leishmania* infection has been observed in both macrophages and T cells [15]. It has been reported that in exudative cell reaction (ECR) lesions, in which phagocytic activity is high, there is increased AnxA1 expression in both M1 and M2 macrophages, while in lesions of the exudative granulomatous reaction (EGR) type, in which the phagocytic rate is reduced, AnxA1 expression is diminished, suggesting that this protein participates in the phagocytosis of *Leishmania* [16,17]. Previous data from our group demonstrated that during infection of BALB/c mice with *L. braziliensis*, higher AnxA1 production coincides with the peak of lesion size and parasitism. An increase in AnxA1 levels was also observed in infected bone marrow-derived macrophages (BMDMs) from WT mice [18]. AnxA1-deficient BALB/c mice, infected with *L. braziliensis,* were able to control tissue parasitism in a similar pattern to WT mice. However, AnxA1-deficient mice presented delayed control of inflammatory responses at late stages of infection, with increased lesion size, more pronounced inflammatory infiltrates, and higher parasite-specific production of IFN-γ, IL-4 and IL-10 [18].

Given the variety of clinical manifestations and host-parasite interaction patterns observed in Leishmaniasis, the role of AnxA1 in this complex set of diseases still deserves further investigation. The present study aimed to unravel the participation of AnxA1 during *L. amazonensis* infection. We found that AnxA1 KO mice are more susceptible to infection. Therapeutic intervention targeting the AnxA1 pathway by using the AnxA1 peptidomimetic Ac2–26 significantly shifted this pattern, improving immune responses and parasite clearance in both WT and AnxA1 KO mice. Altogether, these findings suggest that AnxA1 plays a pivotal role in the regulation of inflammatory responses and parasite clearance during *L. amazonensis* infection.

2. Materials and Methods

2.1. Mice

Female AnxA1 knockout (AnxA1 KO) BALB/c mice, generated as previously described [19], were kindly provided by the Animal Facility of the Department of Biochemistry and Immunology of the Federal University of Minas Gerais (Brazil). WT mice were purchased from the mouse breeding facility of the Federal University of Minas Gerais. The procedures involving animals were in accordance with the National Council on Animal Experiments and Control (Ministry of Science and Technology, Brazil) guidelines. The Animal Ethics Committee of the Federal University of Minas Gerais, Brazil (protocol numbers 240/2016 and 273/2022) approved all described procedures.

2.2. Parasites

L. amazonensis (IFLA/BR/67/PH8) was cultured in Grace's insect medium (Sigma-Aldrich, USA) (pH 6.5) supplemented with 10% heat-inactivated FCS (Gibco), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich) at 25 ℃. Periodically, parasites were recovered from infected mice. All the experiments were conducted with parasites with less than 10 passages in culture [20].

2.3. In vivo L. amazonensis infection and administration of Ac2-26 or WRW4

BALB/c mice (3–6 mice per group) were inoculated in the left hind footpad with 1×10^5 stationary phase promastigotes of *L. amazonensis*. Lesion development was followed weekly with a digital micrometer (Western, Etilux, Brazil). The results were expressed as the difference between measures of infected and noninfected footpads. Mice received an intraperitoneal (i.p) administration of 150 μg/animal of the AnxA1 peptide Ac2–26 [14,21] (GenScript) or 50 μg/animal of the FPR2/3 antagonist WRW4 [22,23] (Calbiochem), twice a week, starting either at two or five weeks after infection up to nine weeks.

2.4. In vitro L. amazonensis infection of bone marrow-derived macrophages and pre-treatment with Ac2-26

Bone marrow-derived macrophages (BMDMs) from BALB/c mice or AnxA1 KO mice were obtained by differentiating bone marrow cells from 6 mice of each group in complete DMEM supplemented with 10% FCS and 20% L-929 culture supernatant as previously described [24]. After 7 days, BMDMs were collected and plated at 1×10^5 cells/well onto a 16-well chamber slide in triplicates. After 2 h of incubation at 37 °C in 5% CO_2 non-adherent cells were removed. After 2 h of pre-incubation with 15 μM of Ac2–26 [25], stationary phase promastigotes of *L. amazonensis* were added to the culture at a multiplicity of infection (MOI) of 5:1 parasite/cell ratio. Macrophage infections were evaluated at 3 and 24 h after infection using Giemsa staining kit (Laborclin, Brazil), according to the manufacturer's instructions. The number of infected and uninfected cells and the number of parasites present in infected cells were determined on an optical microscope. A minimum of 200 macrophages per coverslip was examined.

2.5. Antigen preparation

Early stationary-phase promastigotes from *L. amazonensis* cultures were pelleted by centrifugation and washed twice in PBS. Washed parasites were then resuspended in PBS and disrupted by sonication using 5 cycles of 1 min. The preparation (AgLa) was observed under a microscope for the presence of intact parasites and the protein content was determined by the Bradford method [26].

2.6. Lysate preparation and western blot analysis

Footpads from infected mice were homogenized in complete™ protease inhibitor cocktail (Roche), centrifuged at 11,000 g for 15 min at 4° C and the supernatant was collected and stored at – 20° C for western blot analysis. Western blot analysis was performed as previously described elsewhere [18]. The primary antibodies used were anti-–β-actin (Sigma-Aldrich) and anti-annexin A1 (Invitrogen, Carlsbad, CA). HRP-conjugated anti-rabbit or anti-mouse secondary antibodies were from Cell Signaling Technology. Densitometry analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD). Data were expressed in arbitrary units (AU) after normalization to the of β-actin values.

2.7. Cytokine profile determination

At different time periods post-infection, mice were euthanized, and the draining popliteal lymph nodes were collected. Lymph nodes were processed in a glass homogenizer and the obtained cell suspension had its concentration adjusted to 5×10^6 cells/ml in DMEM supplemented with 10% FCS. The cells were incubated in the absence or presence of stimulus (Ag La: 50 μg/ml) for 48 h at 37 °C, 5% CO_2. ELISA was used to determine the concentration of IFN-γ, TNF-α and IL-10 in supernatants of lymph node cell cultures or BMDMs using the OptEIA™ Mouse Kit (BD Biosciences), according to the manufacturer's instructions.

2.8. Estimation of parasite load

At different periods of time post-infection, mice were euthanized, and the infected paws were collected for parasite load determination by

the limiting-dilution technique, as previously described [27]. Briefly, the infected paws were mechanically homogenized in Grace's insect medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin. Each homogenized sample was serially diluted (1:10) in the same medium (in duplicates). The number of viable parasites was determined from the highest dilution at which promastigotes could be grown for up to 7 days of incubation at 23 °C.

2.9. Histological analysis

The skin samples of the plantar surface of mouse paws were collected and fixed in 10% neutral buffered formalin (pH 7.2). Sampling processes and the determination of inflammatory infiltrate scores were performed as previously described [18].

2.10. Flow cytometry of lymph node cells

Popliteal lymph node cells from WT and KO mice infected with *L. amazonensis* were collected, processed and the cell suspension was labeled for 20 min with different combinations of antibodies: anti-CD45 (clone 30-F11); anti-CD11c (HL3 clone); anti-F4/80 (clone BM8); anti-Ly6G (clone 1 A8-Ly6g); anti-TCRβ (clone H57–597), anti-CD4 (clone GK1.5), anti-CD8 (53–6.7); anti-CD16/32 (clone 2.4G2) and appropriate

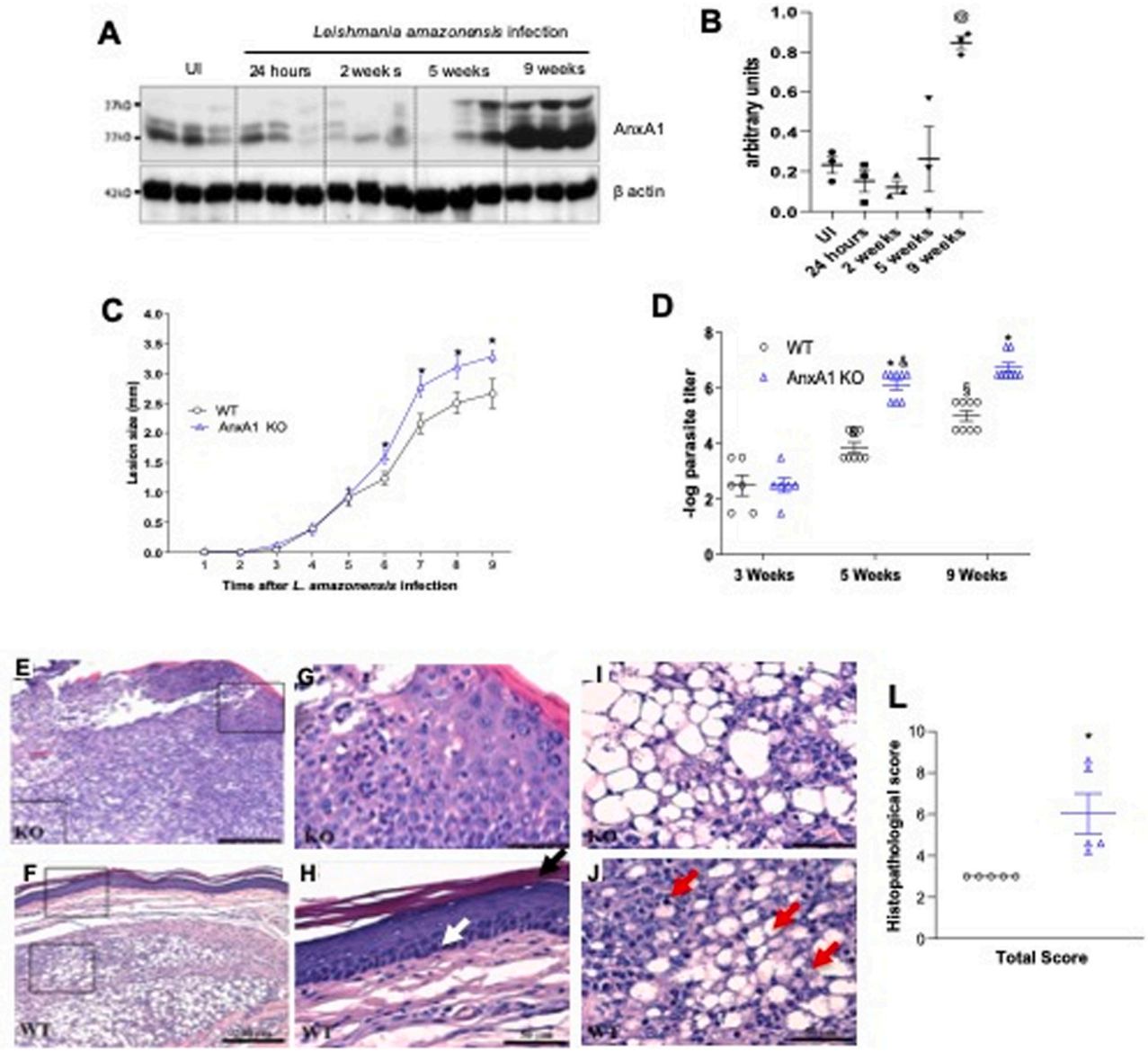

Fig. 1. : Characterization of *L. amazonensis* infection in AnxA1 KO mice. Female BALB/c WT and AnxA1 KO mice were inoculated in the left hind footpad with 1×10^5 promastigotes of *L. amazonensis*. Development of lesions was monitored weekly based on the difference in thickness between the inoculated and the control footpads. Uninfected (UI) and mice infected after 3, 5, 6, and 9 weeks were euthanized. The infected footpads were collected and processed for determination of tissue parasitism, histological analysis and western blotting of AnxA1 expression. **(A)** Western blot analysis of AnxA1, with normalization by β-actin. **(B)** Densitometric analysis of AnxA1 bands after 24 h, 2, 5 and 9 weeks of infection. **(C)** Lesion development. **(D)** Parasite load after 3, 5 and 9 weeks of infection. **(E-J)** Representative histological slides of footpads at 6 weeks of infection. E, G and I (WT); F, H and J (KO). Staining: H&E. E and F: original magnification 20X, bars 200 μm. G-J: original magnification 200X, bars 50 μm. Black arrowhead: acanthosis; (b) white arrowhead: exocytosis; (c) red arrowhead: vacuolated macrophage. **(L)** Histological score of the infected footpads 6 weeks after infection. Western blot and histology data are presented as mean + /- SD of three mice per group at each time point. The course of infection and parasite load data are presented as mean + /- SD of 6 mice per group at each time point. Differences were considered significant when $P \leq 0.05$. * Difference between WT and KO mice at each time-point. $^{\#}$ Difference between infected and uninfected (UI) mice. $^{\&}$ Difference between 3 and 5 weeks. § Difference between 5 and 9 weeks.

negative or isotype controls. For the exclusion of dead cells, the LIVE/DEAD Aqua labeling kit (Thermofisher) was used. At the end of incubation, cells were washed twice and resuspended in PBS with 10% fetal bovine serum. The acquisition was performed on a LSR Fortessa TM (BD Biosciences) flow cytometer. The analyses were performed using the FlowJo software V.10 (BD Biosciences).

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad, USA). Results were expressed as mean ± SD. Kolmogorov-Smirnov normality tests were performed in each group of data with results indicating that all data followed a normal distribution. Data were analyzed using the parametric tests Student t test (when comparing two groups) or One-Way or Two-Way ANOVA followed by Tukey post-test (when comparing three or more groups). The P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. AnxA1 KO mice are more susceptible to L. amazonensis infection

To investigate whether AnxA1 expression is modulated during *L. amazonensis* infection *in vivo*, the kinetics of AnxA1 production in infected WT mice was investigated by Western blotting. The levels of AnxA1 expression coincided with lesion progression in these mice. Upregulation of expression was initiated after five weeks and increased until nine weeks of infection (Fig. 1A-B).

In our previous study we showed that AnxA1 KO mice infected with *L. braziliensis* develop larger lesions but are able to control parasite replication similarly to the WT controls [18]. Here, we asked if the absence of endogenous AnxA1 would have a similar impact on the course of *L. amazonensis* infection. Lack of AnxA1 led to larger lesions in *L. amazonensis* infected mice (Fig. 1C). However, unlike *L. braziliensis* infection, increased parasite burden was also observed in *L. amazonensis*-infected AnxA1 KO mice, as compared to WT mice, at five and nine weeks after infection (Fig. 1D). Histopathological analysis of footpads after six weeks of infection showed that KO mice exhibited increased epidermal exocytosis, acanthosis (Fig. 1H) and the presence of vacuolated macrophages replete with parasites (Fig. 1J), as compared to WT mice. The overall histopathological score was higher in AnxA1 KO as compared to WT mice (Fig. 1L).

3.2. The inflammatory features of L. amazonensis infection are exacerbated in AnxA1 KO mice

To better understand the higher susceptibility of AnxA1 KO mice to *L. amazonensis* infection, we examined the *Leishmania*-specific production by popliteal lymph node of cytokines that are associated with the control of parasite burden and tissue damage. AnxA1 KO mice showed increased *Leishmania*-specific production of IFN-γ by popliteal lymph node cells at five weeks of infection when compared with WT mice (Fig. 2A). Unstimulated cells of AnxA1 KO mice released increased IL-10 levels after five weeks of infection, which was even higher post-antigenic stimulation at five and nine weeks after infection (Fig. 2B). Flow cytometry analysis of popliteal lymph node cells (gating strategy is shown in Fig. 2C) from infected mice showed decreased numbers of $CD4^+$ T cells and increased numbers of $CD8^+$ T cells in AnxA1 KO mice at 3 weeks of infection, while after five weeks this pattern was inverted, showing higher numbers of $CD4^+$ T cells in KO mice. After nine weeks of infection, the numbers of $CD4^+$ T cells were similar between WT and AnxA1 KO mice (Fig. 2D-E). WT and AnxA1 KO mice presented similar numbers of dendritic cells (DCs) in the lymph nodes over time between both genotypes (Fig. 2F). The numbers of macrophages were increased for both groups of mice at 5 weeks after infection, dropping thereafter, at nine weeks (Fig. 2G). Consistent with the non-resolving pattern of *L. amazonensis* infection [28], there were increased numbers of neutrophils over time in both groups of mice, in association with the lesion progression (Fig. 2H).

3.3. Treatment of BMDMs with the AnxA1 peptidomimetic Ac2-26 modulates cytokine production but does not affect the L. amazonensis killing capacity of macrophages

Macrophages play a critical role during *Leishmania* infection and express AnxA1, which has been shown to drive polarization of these cells to resolutive phenotypes [16,29,30]. Therefore, we evaluated macrophage responses to *L. amazonensis* infection *in vitro,* in the context of the absence of endogenous AnxA1, or of its supplementation with its peptidomimetic, named Ac2–26. Thus, BMDMs from WT and AnxA1 KO mice were pre-treated with Ac2–26 for two hours and then infected with *L. amazonensis*. Western blot analysis showed that WT macrophages express AnxA1 during *L. amazonensis* infection (Fig. 3A). The number of parasites per cell after 3 h of infection was smaller in AnxA1 KO macrophages, when compared with WT macrophages, indicating reduced phagocytic activity in the absence of AnxA1 (Fig. 3B). Pre-treatment with Ac2–26 did not modify the phagocytic rates of WT macrophages but was able to restore the phagocytic function of AnxA1 KO macrophages to rates similar to WT macrophages (Fig. 3B). TNF-α levels produced by macrophages of WT and AnxA1 KO mice were similar, after 3 or 24 h of infection. Treatment with Ac2–26 caused an increase in TNF-α production in AnxA1 KO mice after 24 h (Fig. 3C) that, however, did not appear to influence parasite killing (Fig. 3B). Macrophages of AnxA1 KO mice showed higher production of IL-10 when compared with macrophages from WT mice after 3 and 24 h of infection (Fig. 3D). Treatment with Ac2–26 increased IL-10 levels in macrophages from KO mice after 24 h but not in WT macrophages (Fig. 3D).

3.4. Treatment with Ac2-26 reverses the increased susceptibility of AnxA1 KO mice to L. amazonensis infection

Given that the absence of AnxA1 led to increased susceptibility to *L. amazonensis* infection, we asked whether treatment with Ac2–26 could restore AnxA1 signaling and impact the control of inflammatory responses and parasite replication during infection. To test this hypothesis, WT and AnxA1 KO mice were treated with Ac2–26 twice a week for four weeks, starting five weeks after infection with *L. amazonensis* (Fig. 4A). By using this therapeutic protocol, we found that while Ac2–6 treatment did not impact the course of infection in WT mice, AnxA1 KO mice that received Ac2–26 presented smaller lesions when compared with non-treated KO mice (Fig. 4B-C). Reduced lesion size in Ac2–26-treated KO mice was accompanied by increased parasite clearance and diminished tissue damage when compared to non-treated KO mice (Fig. 4D-E). Treatment of WT mice did not have any effect on lesion size and parasite replication (Fig. 4B-D). Higher production of *Leishmania*-specific IFN-γ was found in AnxA1 KO-treated mice when compared to treated-WT mice (Fig. 4F). Treatment with Ac2–26 increased IL-10 levels in WT mice while a decrease is observed in AnxA1 KO mice. *Leishmania*-specific production of IL-10 by lymph node cells was higher for AnxA1 KO mice when compared to WT mice and Ac2–26 administration led to increased IL-10 production in both genotypes (Fig. 4G).

3.5. Early administration of Ac2-26 improves responses to L. amazonensis infection in both WT and KO mice

Since late treatment with Ac2–26 did not modify the course of *L. amazonensis* infection in WT mice, we next questioned whether the infection could be affected by early and prolonged administration of Ac2–26. To address this point, WT, and AnxA1 KO mice received Ac2–26 starting from two weeks up until nine weeks after infection (schematic protocol shown in Fig. 5A). In line with the late protocol, AnxA1 KO

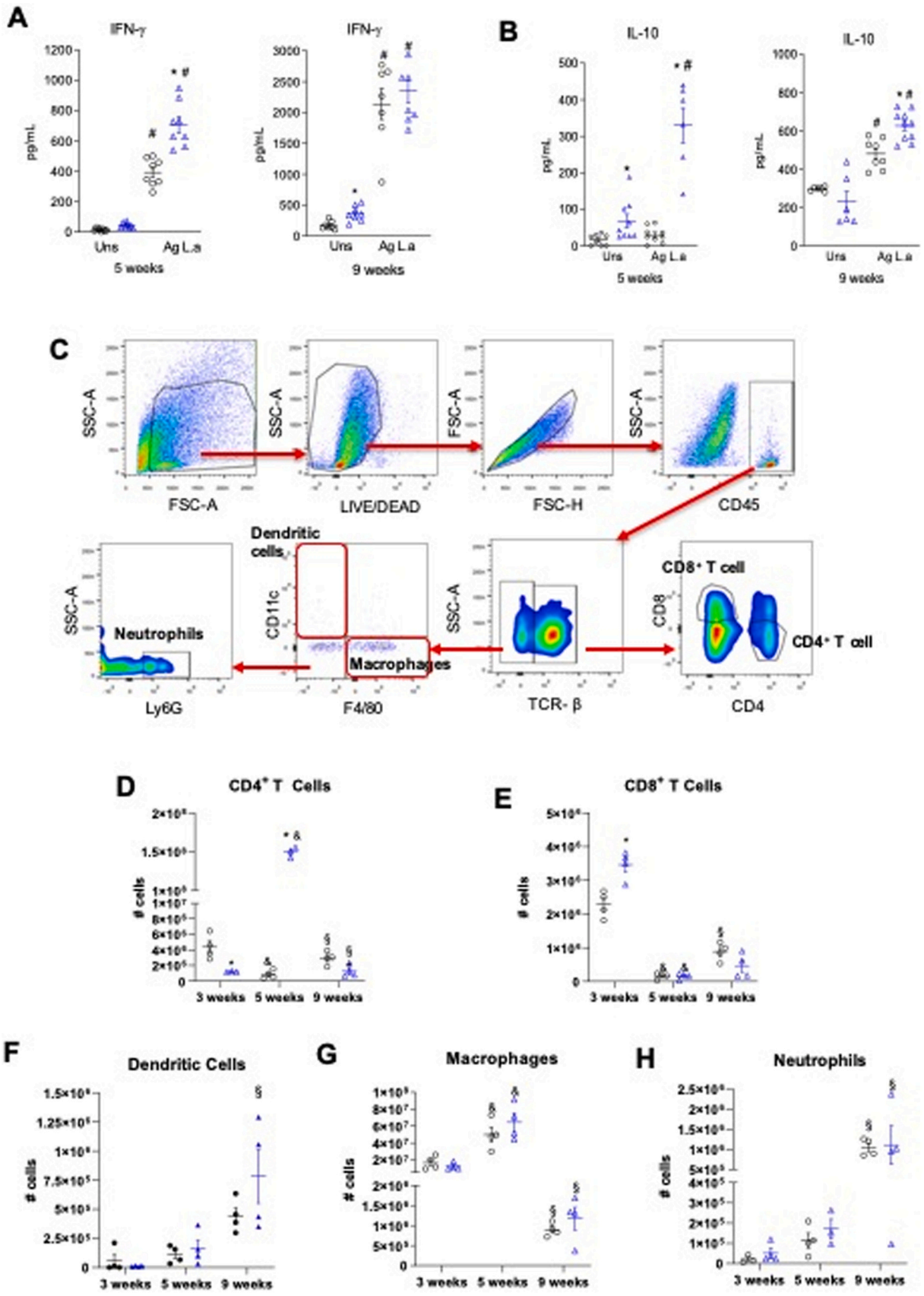

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Fig. 2. : Immune responses during infection of WT and AnxA1 KO mice with *L. amazonensis*. Female BALB/c WT and AnxA1 KO mice were inoculated in the left hind footpad with 1×10^5 promastigotes of *L. amazonensis*. Draining lymph nodes were collected 3, 5 and 9 weeks after infection and cell suspensions were stimulated *in vitro* with particulate antigen of *L. amazonensis* (Ag. La) for 48 h at 37 °C and 5% CO_2. Lymph node cell suspensions were characterized by flow cytometry. IL-10 and IFN-γ were quantified by ELISA. Uns: Unstimulated cells. **(A)** IFN-γ and **(B)** IL-10 production by lymph node cells. **(C)** Gating strategy for flow cytometry experiments. **(D)** Numbers of $CD4^+$ T cells; **(E)** numbers of $CD8^+$ T cells; **(F)** numbers of $CD45^+CD11c^+$ cells (dendritic cells); **(G)** numbers of $CD45^+CD11c^-F4/80^+$ cells (macrophages) and **(H)** numbers of $CD45^+Ly6G^+$ cells (neutrophils) in lymph nodes. All data are presented as mean + /- SD of six mice per group at each time point. Differences were considered significant when $P \leq 0.05$. * Difference between WT and KO mice at each time-point. # Difference between unstimulated and Ag. La-stimulated cells. & Difference between 3 and 5 weeks. § Difference between 5 and 9 weeks.

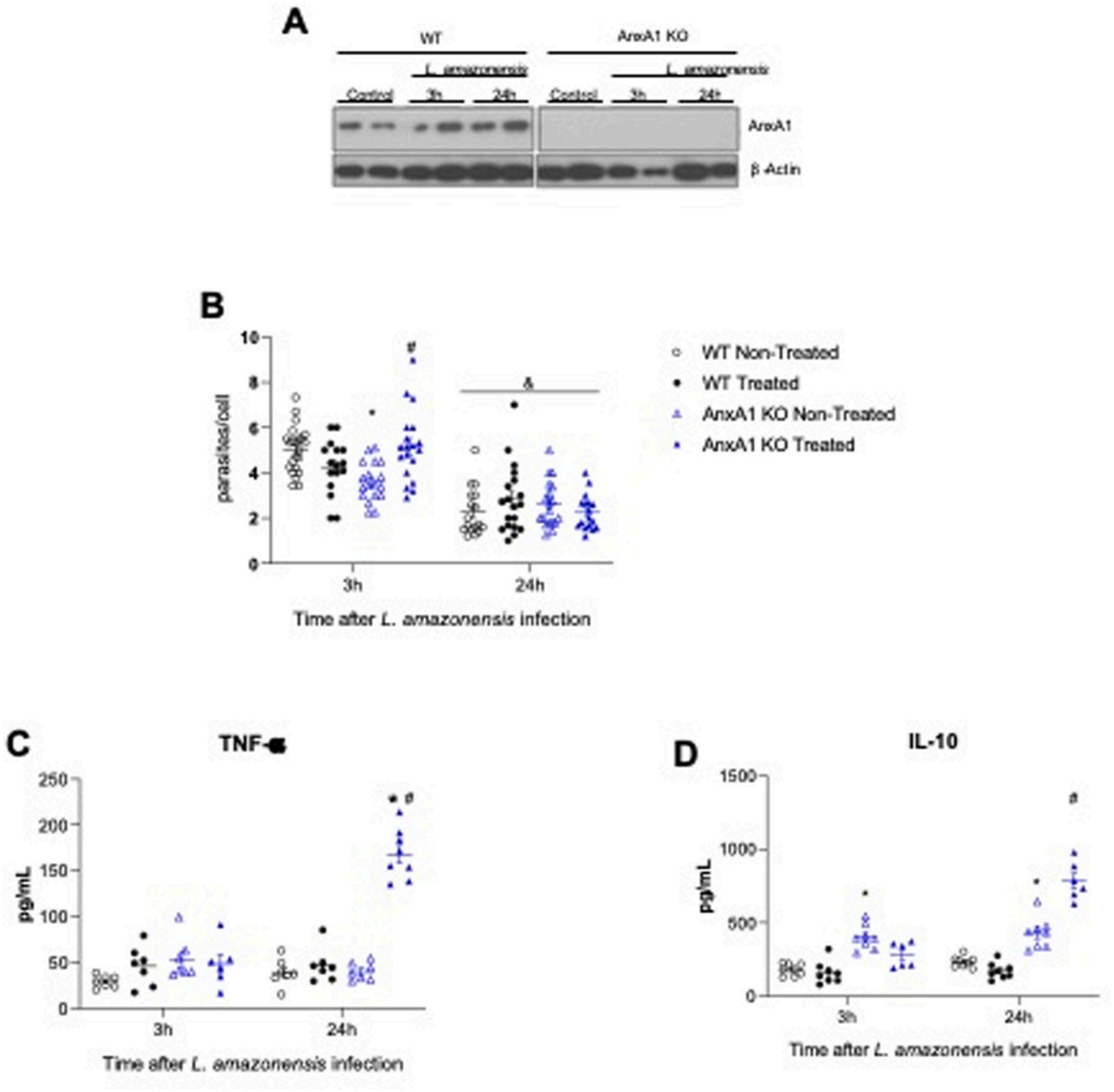

Fig. 3. : Phagocytic activity, immune responses and AnxA1 expression in infected BMDMs of WT and KO mice treated with Ac2–26. BMDMs were pre-treated with Ac2–26 (15 μM) for 2 h and then infected with *L. amazonensis* promastigotes at a 5:1 parasite/cell ratio. At 3- and 24-hours post-infection, cells and culture supernatants were evaluated. **(A)** Western blot analysis of AnxA1, with normalization by β-actin, after 3 and 24 h of infection. **(B)** Number of parasites per infected cell. **(C)** TNF- α production. **(D)** IL-10 production. Data are representative of two experiments with similar results. Differences were considered significant when $P \leq 0.05$. * Difference between WT and KO mice. # Difference between non-treated and treated mice. & Difference between 3 h and 24 h.

mice treated with Ac2–26 showed smaller lesions when compared to non-treated mice and this effect reached significance since the third week of infection (Fig. 5B). Earlier Ac2–26 treatment also reduced parasite burden in the footpads of AnxA1 KO mice (Fig. 5C). Most importantly, for the context of the treatment of an infectious disease, the earlier administration of Ac2–26 to WT-infected mice led to smaller lesions and decreased parasite load when compared to non-treated WT mice (Fig. 5C).

3.6. Blockage of the AnxA1 receptor FPR2 increased susceptibility to L. amazonensis infection in WT mice

Studies have shown that AnxA1 exerts its anti-inflammatory and pro-resolving effects through interaction with the formyl peptide receptor-2 (FPR-2), a G-protein coupled receptor (GPCRs) [31–33]. To investigate the role of AnxA1 signaling in *L. amazonensis* infection, WT mice received WRW4, an antagonist, of FPR2/3 starting five weeks after infection (Fig. 6A). As expected, mice that received WRW4 presented

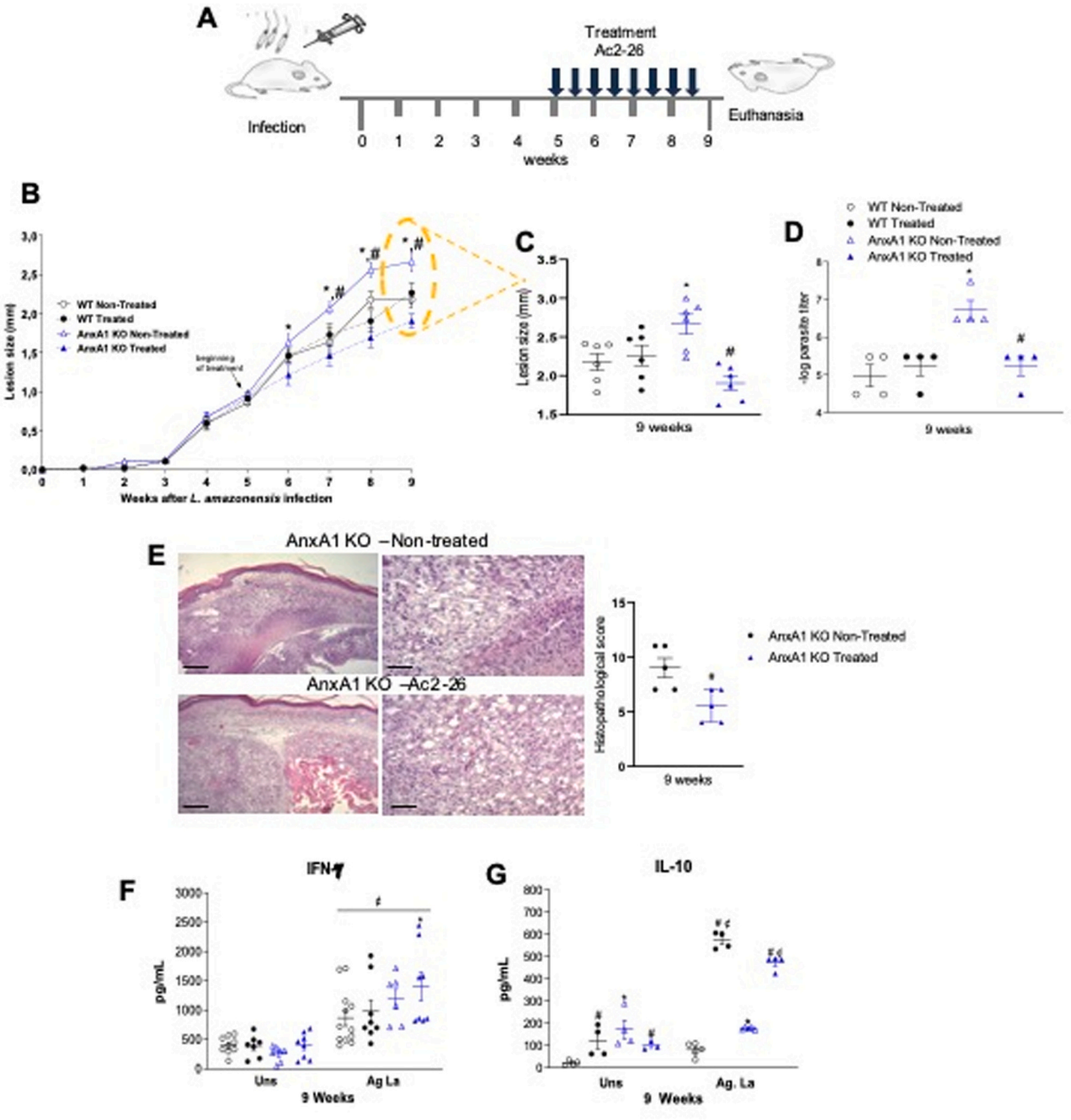

Fig. 4. : Immunopathological consequences of treatment of infected WT and KO mice with the peptide Ac2–26. Female BALB/c WT and ANXA1 KO mice were inoculated in the left hind footpad with 1×10^5 promastigotes of *L. amazonensis*. Animals received the peptide Ac2–26 (150 μg/animal) intraperitoneally twice a week for four weeks starting from the 5th week of infection. After 9 weeks of infection footpads were collected and processed for histology analysis and determination of tissue parasitism. Draining lymph node cells were collected and stimulated *in vitro* with particulate antigen of *L. amazonensis (*Ag. La) for 48 h at 37 °C and 5% CO_2. Uns: Unstimulated cells. IL-10 and IFN-γ levels were quantified by ELISA. **(A)** Graphical scheme of treatment with Ac2–26 starting at the 5th week of infection. **(B)** Lesion development. **(C)** Lesion size at 9 weeks of infection. **(D)** Parasite load after 9 weeks of infection. **(E)** Representative histological slides and histological score of infected paws 9 weeks after infection. Staining: H&E. Left panel: original magnification 20X, bars 200 μm. Right panel: original magnification 200X, bars 20 μm. **(F)** IFN-γ and **(G)** IL-10 levels after 9 weeks of infection. Histology data are presented as mean + /- SD of 3 mice per group at each time point. The course of infection and parasite load data are presented as mean + /- SD of 6 mice per group at each time point. Cytokine levels are presented as mean + /- SD of 8 mice per group at each time point. Differences were considered significant when $P \leq 0.05$. * Difference between WT and KO mice. # Difference between non-treated and treated mice. ¢ Difference between unstimulated and Ag. La-stimulated cells.

increased lesion size (Fig. 6B). However, the late blockage of FPR2/3 signaling had no impact on parasite clearance (Fig. 6C). Administration of WRW4 to WT mice did not affect the levels of IFN-γ secreted by lymph node cells (Fig. 6D) but led to reduced *Leishmania*-specific production of IL-10 (Fig. 6E).

4. Discussion

Distinct profiles of inflammatory responses may be associated with the different tegumentary leishmaniasis clinical outcomes. Among the factors that may affect these distinct patterns, AnxA1 deficiency was shown to exacerbate the inflammatory response in several mice models

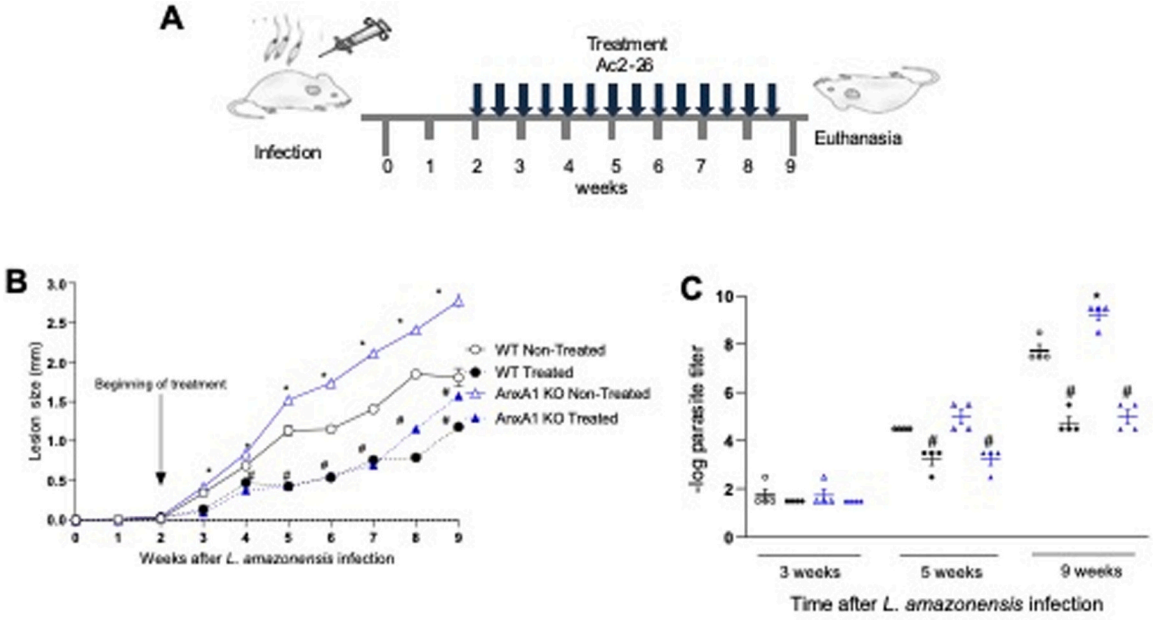

Fig. 5. : Early treatment of infected WT and KO mice with the peptide Ac2–26. Female BALB/c WT and ANXA1 KO mice were inoculated in the left hind footpad with 1×10^5 promastigotes of *L. amazonensis*. Animals received the peptide Ac2–26 (150 μg/animal) intraperitoneally twice a week for seven weeks starting from the 2nd week of infection. After 3, 5 and 9 weeks of infection, footpads were collected and processed for determination of tissue parasitism. **(A)** Graphical scheme of treatment with Ac2–26 starting at the 2nd week of infection. **(B)** Lesion development. **(C)** Parasite load after 3, 5 and 9 weeks of infection. All data are presented as mean + /- SD of 4 mice per group at each time point. Differences were considered significant when $P \leq 0.05$. * Difference between WT and KO mice. # Difference between non-treated and treated mice.

of acute and chronic inflammation [14,19,34], including in *L. braziliensis* infection [18]. However, AnxA1 KO mice can control *L. braziliensis* parasites as well as their WT counterparts, regardless of increased inflammatory responses [18]. On the other hand, the lack of endogenous AnxA1 has been shown to impair microbial clearance in an experimental mouse model of pneumonia [14]. Therefore, we decided to investigate AnxA1 effects on *L. amazonensis-infected* BALB/c mice, a severe model of susceptibility to infection. Here, we show that, in addition to pronounced inflammatory responses, the absence of endogenous AnxA1 further compromises the mice's ability to clear *L. amazonensis*, strengthening evidence that AnxA1 is an important player in controlling inflammatory responses and the outcome of infections by *Leishmania* parasites.

In our study, differences in lesion size and parasitism between WT and AnxA1 KO mice were noticed only after five weeks of infection, sufficient time for the development of an adaptive immune response during an infectious process. Although this pattern seems to be not modified in AnxA1 KO mice, the inflammatory responses and activation of T cell responses in *L. amazonensis* infection were intensified, worsening the infection. In agreement, the analysis of the cellular profile of the lesion-draining lymph nodes revealed a contribution of AnxA1 in determining the components of the inflammatory infiltrate during *L. amazonensis* infection. Significant differences were seen as early as 3 weeks of infection in the numbers of T lymphocytes. Albeit we did not find differences in the numbers of DCs between WT and KO mice, it has been shown that DCs from AnxA1 KO produce lower amounts of IL-12 in response to LPS *in vitro* and have impaired migratory ability when compared with DCs from WT mice [35]. Since uptake of the parasite by DCs and their migration to the lymph nodes are important steps for the generation of a Th1 immune response during *Leishmania* infection [36], it may be suggested that, in the absence of AnxA1, the priming of T cells to a Th1 phenotype is even more delayed.

After five weeks of infection, the absence of AnxA1 promoted significantly higher numbers of $CD4^+$ T cells in the lymph nodes when compared to WT mice. Interestingly, accumulation of $CD4^+$ T cells in lesions of C57BL/6 mice infected with *L. amazonensis* contributes to lesion development [37]. There is also evidence of early recruitment of $CD4^+$ T cells to the lesion site seen in BALB/c mice infected with *L. amazonensis* [38]. Since the differences in lesion size between WT and KO mice start to appear at 5 weeks of infection, the increased numbers of CD4 + T cells may have also a pathogenic role in the context of AnxA1 deficiency in BALB/c mice.

It has been shown that the transfer of macrophages from resistant C57BL/6 mice to lesions of susceptible BALB/c mice led to decreased footpad edema and parasite burden, a response that was associated with higher IFN-γ production by BALB/c mice that received the transfer of cells [39]. These responses may be determinants of the partial protection that is observed in C57BL/6 and suggest that the inability of BALB/c mice to develop and maintain a predominant Th1 response is a driving factor of the susceptibility phenotype. The absence of AnxA1 in our model further skews the balance of the cytokine microenvironment in BALB/c mice, which may therefore contribute to the more severe pathological phenotype observed in AnxA1 KO in our experiments.

Previous studies have looked at the role of AnxA1 on the activation/differentiation of T lymphocytes in murine models and found contrasting results [40–43]. Nonetheless, the differentiation of naïve T cells from AnxA1 null mice *in vitro* to Th1 or Th17 subtypes is impaired [41]. This finding may correlate with our results, since increased IL-10 release is present in WT mice during the initial weeks of infection and production of this cytokine is further exacerbated in AnxA1 KO mice, characterizing a more regulatory response. Interestingly, in Th2-biased conditions, T cells lacking AnxA1 produce not only high amounts of IL-4, but also more IL-10 than T cells from WT mice [41].

AnxA1 KO macrophages have impaired phagocytic capacity, but produce high levels of inflammatory cytokines [44,45], as also shown in *L. braziliensis* infection [18]. *In vitro* stimulation of WT macrophages with the full-length AnxA1 protein induces higher IL-10 levels [11,46]. In contrast, in our study, BMDMs from AnxA1 KO mice produced higher amounts of IL-10 upon infection with *L. amazonensis* when compared to WT mice. Because the anti-inflammatory/pro-resolving effect of other mediators such as lipoxin A4 and resolving D1 can also occur *via* FPR2 [47–49] and stimulation of IL-10 is also a mechanism shared by these

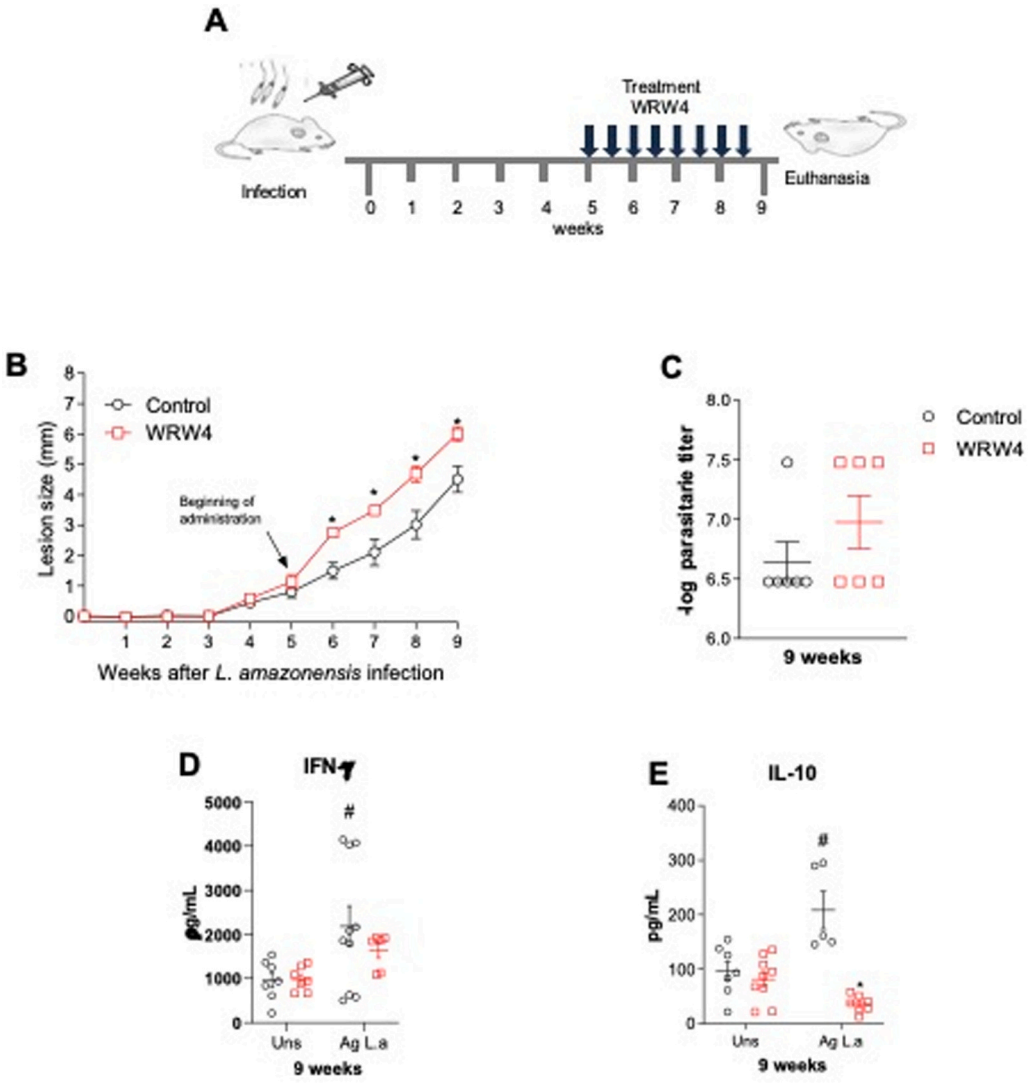

Fig. 6. : Lesion development, tissue parasitism and cytokine levels of infected WT mice after administration of WRW4, an inhibitor of FPR2/3. Female BALB/c WT mice were inoculated in the left hind footpad with 1×10^5 promastigotes of *L. amazonensis*. After five weeks of infection, animals received WRW4 (50 μg/animal) intraperitoneally twice a week for four weeks. After 9 weeks of infection footpads were collected and processed for determination of tissue parasitism. Draining lymph node cells were collected and stimulated *in vitro* with particulate antigen of *L. amazonensis (*Ag. La) for 48 h at 37 °C and 5% CO_2. IL-10 and IFN-γ levels were quantified by ELISA. **(A)** Graphical scheme of WRW4 administration **(B)** Lesion development; **(C)** Parasite load at 9 weeks of infection; **(D)** IL-10 and **(E)** IFN-γ. All data are presented as mean + /- SD of 5 mice per group at each time point. Differences were considered significant when $P \leq 0.05$. * Difference between control and WRW4 mice. # Difference between unstimulated and Ag. La-stimulated cells.

three mediators [46,50,51], it is possible that in AnxA1 KO mice engagement of FPR2 by lipoxin A4 or resolving D1 is potentialized, maintaining the increased amounts of IL-10 observed after *L. amazonensis* infection in mouse BMDMs. Production of IL-10 by *Leishmania*-infected BMDMs can inhibit the secretion of IL-12 and TNF-α by activated macrophages, preventing full activation, which facilitates the replication of the parasite and prevents the development of a protective Th1 immune response [52–54]. If the macrophages from AnxA1 KO mice are also producing higher levels of IL-10 at the site of the infection, this could explain why these mice are less efficient in killing the parasites.

Since AnxA1 deficiency increases *L. amazonensis* susceptibility in BALB/c mice, we decided to investigate whether replacement of this protein in KO mice could ameliorate the responses against the infection. Here, we showed that pre-treatment of BMDMs of AnxA1 KO mice with the peptidomimetic of AnxA1, Ac2–26, improves phagocytosis of *L. amazonensis* parasites and increases cytokine release. Consistent with our observations, Ac2–26 was shown to enhance parasite uptake and cytokine production in macrophages *in vitro* [44], but did not affect the killing capacity of these cells [14].

Administration of recombinant AnxA1 or Ac2–26 to infected mice can improve the clearance of several pathogens, including viruses and protozoan parasites [55–57]. In agreement, our data showed that Ac2–26 treatment enhanced *L. amazonensis* control in AnxA1 deficient mice. More importantly, increasing AnxA1 availability with this peptidomimetic also improved infection outcomes in the AnxA1-sufficient mice. These results are consistent with the known anti-inflammatory/pro-resolving properties of AnxA1, and of its mimetic peptide. Several studies using the administration of recombinant AnxA1 or Ac2–26 have collected data that suggests that the cytokine IL-10 is responsible for at least part of the anti-inflammatory effects of exogenous AnxA1 [46,50,58]. Given that during *L. amazonensis* infection a balance of pro- and anti-inflammatory cytokines in the lesion microenvironment is crucial for determining the host`s ability to control parasite replication while also reducing pathological damage [7,59], the concomitant increased production of both IL-10 and IFN-γ in

Ac2–26-treated AnxA1 KO mice might be shaping the host response to a more resistant phenotype in *L. amazonensis* infection. Furthermore, AnxA1 deficiency has been shown to impair wound healing [60] and treatment with Ac2–26 can induce angiogenesis and collagen deposit, contributing to tissue repair [61,62]. Altogether, these observations suggest that the AnxA1 pathway in tegumentary leishmaniasis may be an alternative target to be further explored as a potential approach for the development of new leishmanicidal drugs.

As a proof-of-concept and to further explore this pathway as a promising pharmacological intervention, we tested whether the administration of WRW4, an antagonist of FPR2 that also inhibits FPR3, to BALB/c mice would impact the infection. Inhibition of FPR2 with WRW4 in different pre-clinical mouse models can increase susceptibility to infections and has been shown to prevent the protection against exacerbated inflammation that is conferred by the administration of Ac2–26, highlighting the importance of this receptor for the effects of AnxA1 [21,63–65]. Injection of WRW4 did not affect tissue parasitism but led to increased lesion size in *L. amazonensis* infection, which could be associated with the diminished IL-10 production. This effect was seen right after the beginning of the administration of WRW4. Although WRW4 is a potent inhibitor of both FPR2 and FPR3 [66], FPR3 is less commonly expressed than FPR2, is localized in intracellular vesicles, not membrane-bound, and has only one known selective, high-affinity ligand [67,68]. Therefore, FPR2 and not FPR3 may be the most likely responsible for the differences seen in our experiments. Coupled with our findings, we suggest that the anti-inflammatory properties of the AnxA1-FPR2 signaling pathway act throughout the whole course of *Leishmania* infection and reinforce its role in modulating infection outcomes.

Finally, it is noteworthy that the results presented here are the first to show that AnxA1 contributes to the susceptibility phenotype of BALB/c during *L. amazonensis* infection and the potential clinical applications of targeting the AnxA1 pathway with administration of Ac2–26. Further studies are needed to advance our knowledge of the mechanisms by which AnxA1 impacts the immune responses to this parasite and to better explore this pathway as a potential therapeutic target for *Leishmania* infections. Nonetheless, the reported findings bring an interesting prospect for the treatment of tegumentary leishmaniasis, a neglected disease for which few therapeutic options are available.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee (CEUA) of the Federal University of Minas Gerais of the Federal University of Minas Gerais, Brazil under the protocol numbers 240/2016 and 273/2022.

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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.

Data availability

Data will be made available on request.

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