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Annexin A1 Is Involved in the Resolution of Inflammatory Responses during *Leishmania braziliensis* Infection

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Leishmaniases are diseases caused by several *Leishmania* species. *Leishmania* (*Viannia*) *braziliensis* can cause localized cutaneous leishmaniasis (LCL), which heals spontaneously, or mucosal leishmaniasis (ML), characterized by chronic and intense inflammation and scanty parasitism. Annexin A1 (AnxA1) is a protein involved in modulation and resolution of inflammation through multiple mechanisms. In the present study, the role of AnxA1 was investigated in *L. braziliensis*–infected BALB/c mice. AnxA1 levels increased at the peak of tissue lesion and parasitism in infected mice. AnxA1 increased also after *L. braziliensis* infection of BALB/c (wild-type [WT]) bone marrow derived macrophages. Despite a lower parasite intake, parasite burden in bone marrow-derived macrophages from AnxA1$^{-/-}$ mice was similar to WT and associated with an early increase of TNF-α and, later, of IL-10. AnxA1$^{-/-}$ mice controlled tissue parasitism similarly to WT animals, but they developed significantly larger lesions at later stages of infection, with a more pronounced inflammatory infiltrate and increased specific production of IFN-γ, IL-4, and IL-10. AnxA1$^{-/-}$ mice also presented higher phosphorylation levels of ERK-1/2 and p65/RelA (NF-κB) and inducible NO synthase expression, suggesting that AnxA1 may be involved in modulation of inflammation in this model of experimental leishmaniasis. Finally, assessment of AnxA1 levels in sera from patients with LCL or ML revealed that ML patients had higher levels of serum AnxA1 than did LCL patients or control subjects. Collectively, these data indicate that AnxA1 is actively expressed during *L. braziliensis* infection. In the absence of AnxA1, mice are fully able to control parasite replication, but they present more intense inflammatory responses and delayed ability to resolve their lesion size. ***The Journal of Immunology*****, 2017, 198: 3227–3236.**

Protozoa of the genus *Leishmania* are intracellular parasites of a phagocytic mononuclear system, which are transmitted to mammalian hosts by the bite of a sand fly vector (1). Approximately a third of the 1.2 million new cases of tegumentary leishmaniasis reported worldwide occur in the Americas, and Brazil is 1 of the 10 countries with the highest incidence rates (2). Therapeutic alternatives for treatment of tegumentary leishmaniasis are scant, toxic, and per se not effective (3).

Leishmania (*Viannia*) *braziliensis* is the most prevalent species in Latin American countries and may cause localized cutaneous leishmaniasis (LCL) or mucosal leishmaniasis (ML). In LCL, skin ulcerative lesions develop at the site of a sand fly bite, which may heal spontaneously or persist for years. ML is a chronic condition that leads to destruction of mucosal tissues, extending from the nose and mouth to the pharynx and larynx (4). In both clinical conditions, increased production of proinflammatory cytokines, including IFN-γ and TNF-α, is essential for parasite control (4, 5). However, in ML, they are also responsible for tissue damage observed at late stages of disease due to exacerbated activation of type 1–specific T lymphocytes (3, 6). Therefore, ambiguously, scant parasites and intense inflammatory responses coexist and may persist for long periods in ML lesions (7–9), leading to unresolved inflammation, failure in parasite clearance, exacerbated host tissue damage, and abnormal repair.

Resolution of inflammation is an active process that requires production of anti-inflammatory and proresolving molecules, a

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Abbreviations used in this article: AgLb, *Leishmania braziliensis* Ag; AnxA1, annexin A1; AU, arbitrary unit; BMMϕ, bone marrow–derived Mϕ; iNOS, inducible NO synthase; LCL, localized cutaneous leishmaniasis; Mϕ, macrophage; ML, mucosal leishmaniasis; WT, wild-type.

switch in the proteic and lipid mediators class from pro- to anti-inflammatory, apoptosis of granulocytes with subsequent removal by macrophages (Mϕs), and change of Mϕ phenotype to a more restorative one (10–12). Proresolving mediators are specialized molecules, which activate resolution pathways to return the tissue to homeostasis. Functionally, they can act to limit additional leukocyte recruitment, induce neutrophil apoptosis and Mϕ reprogramming, enhance efferocytosis by Mϕs, promote the return of nonapoptotic cells to the lymphatics, and to start tissue repair mechanisms, helping to resolve infectious disease. These mediators are diverse in nature and include specialized lipid mediators (lipoxins, resolvins, protectins, and maresins), proteins and peptides (annexin A1 [AnxA1], galectins), gaseous mediators (hydrogen sulphide), and purine (adenosine) (13, 14).

AnxA1 is a 37-kDa calcium-dependent phospholipid-binding protein, regulated by glucocorticoids, which binds to and activates the formyl peptide receptor 2/lipoxin A_4 receptor FPR2/ALXR (15) and has significant roles in modulation of the inflammatory response (16–21). AnxA1 regulates the inflammatory response through modulation of the generation of proinflammatory mediators, including those derived from activation of phospholipase 2, cyclooxygenase-2, and inducible NO synthase (iNOS), as well as of the anti-inflammatory cytokine IL-10. Recent evidence suggests that AnxA1 may control leukocyte apoptosis and favor their removal by Mϕ phagocytosis (22–25). Additonally, T cell–expressed AnxA1 has significant effects in T cell–driven inflammatory responses, including T cell–intrinsic intracellular signaling, proliferation, and Th1/Th17 cytokine release (26). In intestinal mucosal wounds, endogenous AnxA1 is also released by extracellular vesicles derived from epithelial cells, activating repair circuits (27).

Although the role of AnxA1 in various inflammatory conditions has been fairly investigated, its involvement in infection processes has been poorly addressed, especially in parasite infection (28–30). We have, accordingly, hypothesized that AnxA1 may be important for the control of inflammatory response in *L. braziliensis* infection. Therefore, this study sought to compare the kinetics of inflammatory and proresolving responses during the course of *L. braziliensis* infection in wild-type (WT) BALB/c and AnxA1-deficient mice. The BALB/c mouse is resistant to *L. braziliensis* infection (9), controlling both the inflammatory response and parasite replication, thus being a suitable model to investigate resolution of inflammation in *Leishmania* infection.

Materials and Methods

Mice

Female AnxA1 knockout ($AnxA1^{-/-}$) mice on a BALB/c background, generated as previously described (31), and WT BALB/c mice were obtained from the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) breeding unit. Animals were given water and food ad libitum. The procedures involving animals were in accordance with the National Council on Animal Experiments and Control (Ministry of Science and Technology, Brazil) guidelines. All described procedures had prior approval from the Animal Ethics Committee of the Federal University of Minas Gerais, Brazil (National Council on Animal Experiments and Control/Federal University of Minas Gerais protocol nos. 15/2011 and 221/2007).

Parasites

L. (*Viannia*) *braziliensis* (MHOM/BR/75/M2903 strain) was cultured in Grace's insect medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS (LGC Biotecnologia, Cotia, SP, Brazil), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) (pH 6.5) at 25 ± 1°C. Parasites were periodically recovered from infected animals and subcultured every 3 d at 1×10^5 parasites/ml. All experiments were conducted with parasites with fewer than 15 passages in culture (32).

Infection

Mice were inoculated in the left hind footpad with 1×10^7 early stationary phase promastigotes of *L. braziliensis*, and lesion development was followed weekly with a digital micrometer (Western, Etilux, São Paulo, SP, Brazil). The results were expressed as the difference between measures of infected and noninfected footpad (33).

In vitro L. braziliensis *infection in bone marrow–derived Mϕs*

Bone marrow–derived Mϕs (BMMϕs) from BALB/c WT or $AnxA1^{-/-}$ mice were obtained as previously described (34). BMMϕs were plated at 5×10^6 cells/ml onto round coverslips in DMEM (Sigma-Aldrich) containing 10% FCS, 1 mM L-glutamine, 100 U/ml penicillin G potassium, 25 mM HEPES (Sigma-Aldrich), and 50 mM 2-ME (Pharmacia Biotech, Uppsala, Sweden) in 24-well plates. Cells were incubated for 90 min at 37°C in 5% CO_2. Nonadherent cells were removed by washing with PBS. *L. braziliensis* promastigotes were added to the culture at a 5:1 parasite/cell ratio. After 3 h, cells were washed with PBS to remove noninternalized parasites. Coverslips were collected to evaluate infectivity after 3, 24, and 48 h of incubation. Culture supernatants were also collected for cytokine measurements and maintained at −80°C until use. Coverslips were stained by May–Grünwald–Giemsa using a kit (Laborclin, Pinhais, PR, Brazil), according to the manufacturer's instructions. The analysis was performed using an Olympus BX50 optical microscope (Olympus, Center Valley, PA). The number of infected and uninfected cells and the number of parasites present in infected cells were determined. A minimum of 200 Mϕs per coverslip were examined.

Ag preparation

Promastigotes from early stationary phase cultures were washed twice in PBS. The pellets obtained were submitted to seven cycles of freezing in liquid nitrogen followed by thawing at 37°C. The preparations were observed under a microscope for the presence of intact parasites (33). Protein content was determined by Bradford method (35) and adjusted to 1 mg/ml. The Ag suspension was stored at −80°C in aliquots.

Lysate preparation and Western blot analysis

Infected paws were collected and footpads were isolated. Tissue fragments were added to a lysis solution consisting of 1% Nonidet P-40, 100 mM Tris/HCl (pH 8), 10% glycerol, 5 mM EDTA, 1 mM $NaVO_3$, 1 mM DTT, 1 mM PMSF, 200 mM NaCl, 25 mM NaF, leupeptin, and aprotinin. The tissues were then homogenized in an Ultra-Turrax Power Gen 1000 (Fisher Scientific/IKA, Zuideinde, the Netherlands) and incubated on ice for 15 min. Finally, the lysates was centrifuged (12,000 rpm, 15 min at 4°C) and the supernatant aliquots were stored at −20°C until use. The same lysis solution was used to prepare lysates from *L. braziliensis*–infected BMMϕs. The Bradford assay reagent (Bio-Rad Laboratories) was used to quantify proteins. Western blot analysis was as described (25). Extracts (50 μg) were separated by electrophoresis on a denaturing 10–15% polyacrylamide-SDS gel and electrotransferred to nitrocellulose membranes. Then, membranes were blocked for 1 h at room temperature with PBS containing 5% (w/v) nonfat dry milk and 0.1% Tween 20, washed three times with PBS containing 0.1% Tween 20, and then incubated with specific primary Abs at a dilution of 1:1000 overnight: anti-AnxA1 (Invitrogen, Carlsbad, CA), anti–β-actin (Sigma-Aldrich), anti-iNOS (Santa Cruz Biotechnology, Dallas, TX), cleaved anti–caspase-3, anti–p-ERK1/2, and anti–p-NF-κBp65/RelA (the last three from Cell Signaling Technology, Danvers, MA). After washing, membranes were incubated with appropriated HRP-conjugated secondary Ab (1:3000). Immunoreactive bands were visualized by using an ECL detection system, as described by the manufacturer. Densitometry analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD). Data were expressed in arbitrary units (AU) after normalization to the values of β-actin in the same sample.

In vitro stimulation of mononuclear cells and quantification of parasites

At different periods of time postinfection, animals were euthanized. The infected paw and the draining popliteal lymph nodes were collected. Lymph nodes were processed in a glass homogenizer. The obtained cell suspension had its concentration adjusted to 5×10^6 cells/ml in complete tissue culture medium and was stimulated with *L. braziliensis* Ag (AgLb; 50 μg/ml). Complete tissue culture medium was used as a negative control (36). The supernatant was collected after 48 h of incubation at 37°C, 5% CO_2, and stored at −20°C for cytokine assays (37). To quantify parasites, we used a limiting dilution technique (32). Briefly, the infected paws were processed

on a glass homogenizer in Grace's medium (pH 6.5). For debris removal, the obtained suspension was centrifuged at 50 × *g* at 4°C for 1 min. The supernatant was collected and centrifuged at 1540 × *g* at 4°C for 10 min. The pellet obtained was resuspended in Grace's medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μl/ml streptomycin. The parasite suspension was subjected to serial dilution 1:10 in 96-well culture plates. After incubation at 25°C for 15 d, the wells were evaluated for the presence of parasites. Results were expressed as the negative logarithm titer of parasites, which corresponds to the last dilution at which viable parasites were observed (32).

ELISA for cytokines and AnxA1 quantification

Quantification of cytokines in culture supernatants was performed by ELISA. BD OptEIA (BD Biosciences, San Diego, CA) kits were used for measurement of TNF-α, IFN-γ, IL-4, and IL-10, according to the manufacturer's guidelines. Levels of AnxA1 in serum of patients were determined using an ELISA kit for AnxA1 (USCN Life Sciences, Houston, TX), according to the manufacturer's instructions.

Histological analysis

The skin samples of the plantar surface of mouse paws were collected and fixed in 10% neutral buffered formalin (pH 7.2). These samples were processed for routine histology, sectioned at 5 μm, and stained with H&E. The inflammatory reaction was evaluated using a semiquantitative procedure assessing the presence of leukocytes in the dermis and hypodermis. The inflammatory infiltrate score system was adapted from Alves et al. (38), as follows: 0, absent, no inflammatory infiltrate (histologically healthy dermis); 1, slight, diffuse inflammatory infiltrate, especially in the upper dermis (1–9 cells per field/fields); 2, moderate, diffuse or focal inflammatory infiltrate, including areas in the deep dermis or hypodermis (10–30 cells per field/20 fields); and 3, intense, severe diffuse or focal inflammatory infiltrate around the vessels, glands, hair follicles in deep dermis and/or hypodermis (>30 cells per field/20 fields). Histopathological analysis was performed using a single-blinded model.

Human sera

This study was conducted according to the Declaration of Helsinki principles. The Ethics Committee from the Federal University of Minas Gerais (Belo Horizonte, Minas Gerais, Brazil; protocol no. CAAE-323431 14.9.0000.5149) approved the study. All control subjects and patients received an individual copy of the study policy, which was reviewed by an independent person, and all participants provided their consent form in Portuguese, before the collection of their blood sample. Sera samples of control subjects ($n = 10$; including 5 males and 5 females, with ages ranging from 9 to 35 y, to pair with LCL patients; and $n = 12$, 10 males and 2 females, with ages ranging from 25 to 76 y, to pair with ML patients) and patients with a confirmed diagnosis of LCL ($n = 10$; including 5 males and 5 females, with ages ranging from 9 to 33 y) or ML ($n = 12$; including 10 males and 2 females, with ages ranging from 29 to 74 y), from an endemic area of leishmaniasis (Belo Horizonte, Minas Gerais, Brazil), were used. The diagnosis was confirmed by clinical evaluation of lesions, which were compatible with either LCL or ML, as well as by direct demonstration of parasites in Giemsa-stained smears of biopsies of skin (LCL) or mucosal fragments (ML) of the patients. Additionally, analyses from two biopsy samples collected from each patient were submitted to a conventional PCR technique, and they presented positive results for *L. braziliensis* DNA. None of the patients had been previously treated with antileishmanial drugs before the sample collection. All patients in this study were integrated into the clinical assistance Program of the Clinical Hospital from the Federal University of Minas Gerais, and they received continuous medical assistance. All samples were collected by venipuncture of medial vein in tubes without anticoagulant, and were kept at 37°C by 15 min, when they were centrifuged at 4000 × *g* for 15 min. Sera were separated and kept at −80°C until use.

Statistical analysis

Results were expressed as mean ± SD or as mean ± SEM, as indicated in each figure legend. Data were analyzed by ANOVA followed by Newman–Keuls posttest or a Student *t* test, according to the characteristics of each experiment, using GraphPad Prism software, version 5.0.3 (GraphPad Software, La Jolla, CA). A p value <0.05 was considered significant.

Results

AnxA1 is modulated during the course of L. braziliensis *infection in BALB/c mice and in vitro in BMMϕs*

Because the expression of AnxA1 in a *Leishmania* infectious model has not been previously investigated, we first evaluated the kinetics of AnxA1 expression during the course of *L. braziliensis* infection in BALB/c mice. As shown in Fig. 1A, *L. braziliensis* infection in WT BALB/c mice followed a pattern of resistance to infection, as edema at the infection site increased in size, reaching a maximum average value on the second week postinfection, and decreased gradually afterward. Tissue parasitism correlated with lesion size, as high numbers of parasites were detected on the second week postinfection, but decreased progressively from the second week on (Fig. 1B). Parasites were no longer detected at 8 wk postinfection.

Following the infection course, AnxA1 expression in WT animals increased as early as 1 d after *L. braziliensis* infection, remained high for 2 wk, when higher lesion size and parasitism peaks were observed, but decreased, alongside the decrease in lesion size and tissue parasitism, reaching significantly lower levels at 12 wk postinfection (Fig. 1C; corresponding densitometry analysis in Fig. 1D). These findings indicate that AnxA1 expression is induced during *L. braziliensis* infection in BALB/c mice, and it may be important for control of the inflammatory process in this model of resistance to infection, because their levels were up- and downregulated in parallel with lesion size and parasitism. *L. braziliensis* infection also induced phosphorylation of ERK1/2 and p65/RelA, as compared with the noninfected group (data not shown).

Because Mϕs are the primary target of intracellular *Leishmania* infection and AnxA1 is known to modulate dendritic cell and Mϕ functions, including phagocytic competence (39, 40), we investigated

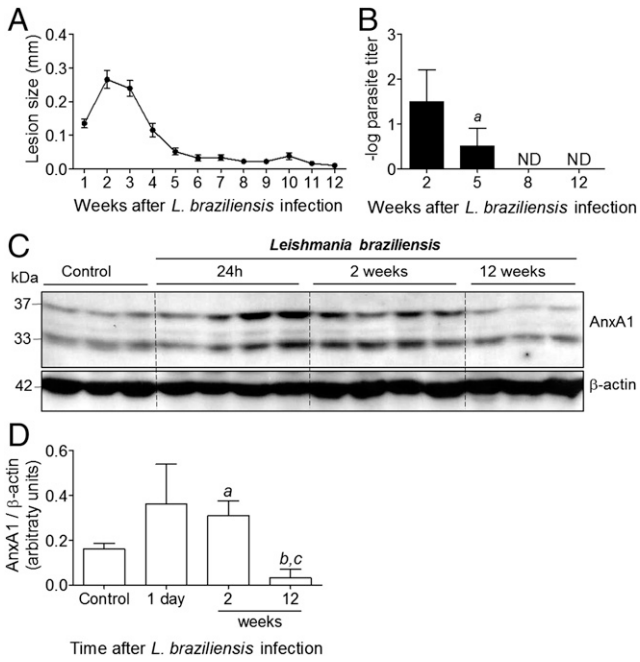

FIGURE 1. AnxA1 expression during the course of *L. braziliensis* infection in BALB/c mice. BALB/c mice were infected in the left hind footpad with 1×10^7 promastigotes of *L. braziliensis*. (**A**) Lesion sizes were monitored weekly with a digital caliper. Data are presented as mean ± SEM. (**B**) Parasite loads were evaluated at different times after infection. Statistically significant differences were assumed when $p < 0.05$ in relation to (*a*) 2 wk postinfection. Data are presented as mean ± SD of at least four mice in each time point (**C**) Western blotting analysis of AnxA1. For loading control, membranes were reprobed with β-actin. (**D**) Densitometric analysis of AnxA1 bands normalized to β-actin. Data are presented as mean ± SD of at least three mice in each time point. Data are representative of two experiments with similar results. Statistically significant differences were assumed when $p < 0.05$ in relation to (*a*) control noninfected animals, (*b*) 1 d postinfection, and (*c*) 2 wk postinfection. ND, not detected.

the kinetics of AnxA1 expression in BMMϕs from WT mice and the phagocytic capacity of Mϕs derived from AnxA1$^{-/-}$ mice. As previously demonstrated in our in vivo assays in BALB/c mice, AnxA1 expression was also modulated in WT mice BMMϕs, increasing significantly after 24 h of infection (Fig. 2A, 2B). Although the proportion of infected BMMϕs was similar between WT and AnxA1$^{-/-}$ mice (Fig. 2C), AnxA1$^{-/-}$ BMMϕs displayed lower numbers of parasites per infected cell shortly after infection (3 h), suggesting an impaired ability of phagocytosis in AnxA1$^{-/-}$ mice. However, as seen in Fig. 2D, after 24 h of infection, similar numbers of parasites per infected cell were observed in both groups, indicating that Mϕ activity in response to infection was not affected. At the 48 h time point, the numbers of parasites are smaller in WT cells, whereas they remain constant in AnxA1$^{-/-}$ cells, as compared with 3 h postinfection. Interestingly, *L. braziliensis*–infected BMMϕs derived from AnxA1$^{-/-}$ mice produced more TNF-α after 3 and 24 h of infection, and IL-10, after 24 and 48 h, as compared with BMMϕs derived from WT mice (Fig. 2E, 2F). Collectively, these findings suggest that AnxA1 deficiency impacts Mϕ competence for *L. braziliensis* phagocytosis, but it does not impair the fight against parasites, at least in part due to greater TNF-α production.

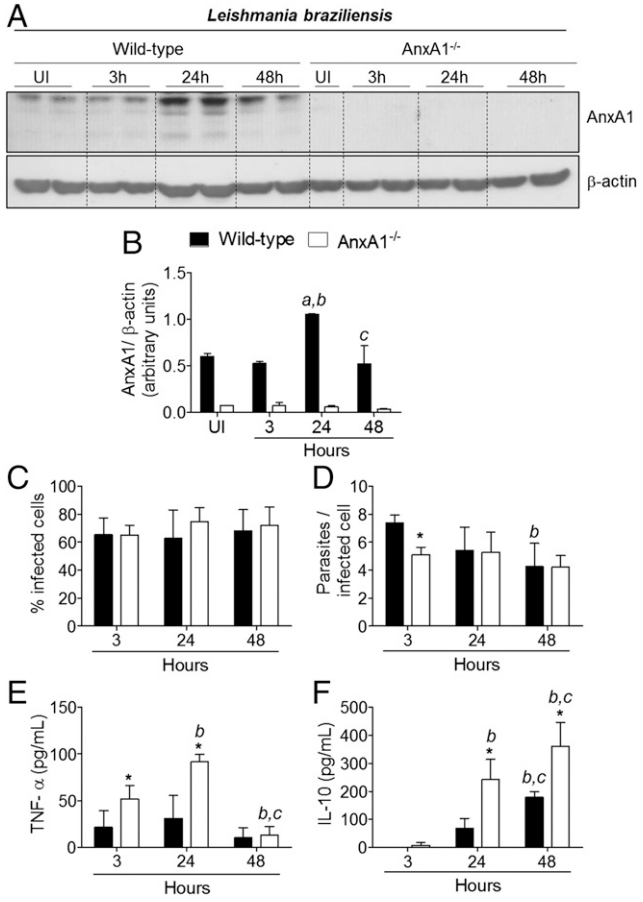

FIGURE 2. AnxA1 expression in BALB/c BMMϕs. BMMϕs from WT BALB/c or AnxA1$^{-/-}$ mice were infected with *L. braziliensis* promastigotes, at a 5:1 parasite/cell ratio. (**A** and **B**) AnxA1 deficiency and absence of unspecific reaction with parasites proteins were confirmed by Western blotting in uninfected cells (UI) and in cells infected during 3, 24, and 48 h. For loading control, membranes were reprobed with β-actin. Blots are representative of three experiments using pooled cells from at least two animals in each experiment. At 3, 24, and 48 h postinfection, cells were evaluated to (**C**) percentage of infected cells and (**D**) number of parasites per infected cells. At the same time points, supernatant was assessed to TNF-α (**E**) and IL-10 (**F**) concentrations. Data are presented as mean ± SD. Data are representative of two experiments with similar results, with at least four mice per group in each time point. Statistically significant differences were assumed when $p < 0.05$ in relation to (*a*) uninfected cells, (*b*) 3 h postinfection, and (*c*) 24 h postinfection. *$p < 0.05$ between WT and AnxA1$^{-/-}$ mice.

AnxA1$^{-/-}$ mice effectively control parasite multiplication but display increased lesion size and inflammatory infiltrates during L. braziliensis *infection as compared with WT mice*

Given that AnxA1 levels were modulated in vivo and in vitro during the course of *L. braziliensis* infection in BALB/c mice, we compared the edema and the tissue parasitism in WT BALB/c and AnxA1$^{-/-}$ mice. As seen in the Fig. 3A, the pattern of lesion size development in AnxA1$^{-/-}$ mice was similar to that observed in WT animals. However, AnxA1$^{-/-}$ mice display further and significantly increased lesion size at the third week postinfection, as compared with WT mice, suggesting a delay in the resolution of inflammatory responses. We also investigated the ability of AnxA1$^{-/-}$ to control parasite replication at the infection site. Comparable numbers of parasites were detected in AnxA1$^{-/-}$ and WT mice either at 2 or 5 wk postinfection (Fig. 3B), indicating that the lack of AnxA1 did not affect the control of tissue parasitism in *L. braziliensis* infection.

The histopathological analysis of skin biopsies of footpads of *L. braziliensis*–infected mice revealed significant inflammatory alterations, especially in the deep dermis and hypodermis (Fig. 3C, 3E). Significant epidermal changes, including acanthosis, dyskeratosis, papillomatosis, exocytosis, or hyperkeratosis, have not been detected either in WT or in AnxA1$^{-/-}$ infected samples. In contrast, *L. braziliensis* infection for 4 wk triggered evident inflammatory cell infiltration into deep dermis and/or hypodermis. Whereas WT mice presented slight to moderate leukocyte infiltration (Fig. 3C, 3D), AnxA1$^{-/-}$ mice had moderate to severe leukocyte infiltration (Fig. 3E, 3F). A mixed population of mononuclear (Mϕs, plasma cells, and lymphocytes) and polymorphonuclear (neutrophils and eosinophils) leukocytes characterized the cellular exudates of the WT and AnxA1$^{-/-}$ mice. The distribution of the inflammatory infiltrate varied from focal to diffuse exudates, with the diffuse pattern more frequent, along the deep dermis, hypodermis, and associated muscle tissue (Fig. 3C, 3F). Quantification of the histopathological findings is presented in Fig. 3G. Other histological events potentially associated with *L. braziliensis* skin infection, such as the presence of Langham's giant cells or foreign body giant cells, typical granulomatous chronic inflammatory reaction, or necrotic areas, have not been observed. At 8 wk postinfection, inflammatory responses decreased in both groups of mice. Nevertheless, no significant differences in the inflammatory cell infiltration were detected in WT and AnxA1$^{-/-}$ mice, suggesting that, regardless of the lack of AnxA1, inflammatory reactions tended to resolve in all animals.

Following the evidence that AnxA1$^{-/-}$ mice efficiently controlled parasite multiplication, but displayed delayed resolution of inflammatory response, the levels of some key cytokines that are essential for activation of Mϕ leishmanicidal mechanisms and control of *L. braziliensis* infection were investigated (Fig. 4). IFN-γ (Fig. 4A), IL-4 (Fig. 4B), and IL-10 (Fig. 4C) levels produced by lymph node cells were significantly increased in AnxA1$^{-/-}$ mice at 2 wk postinfection in response to AgLb, as compared with WT animals. After 6 wk of infection, IFN-γ production in WT mice returned to basal levels, although it remained higher in AnxA1$^{-/-}$ mice. At this time point, the levels of IL-4 and IL-10 were not different between the two genotypes. These findings may also indicate that the increased IFN-γ levels were associated with more intense edema and inflammatory responses in AnxA1$^{-/-}$ mice. Alternatively, the concomitant increase in IL-4 and IL-10 levels in

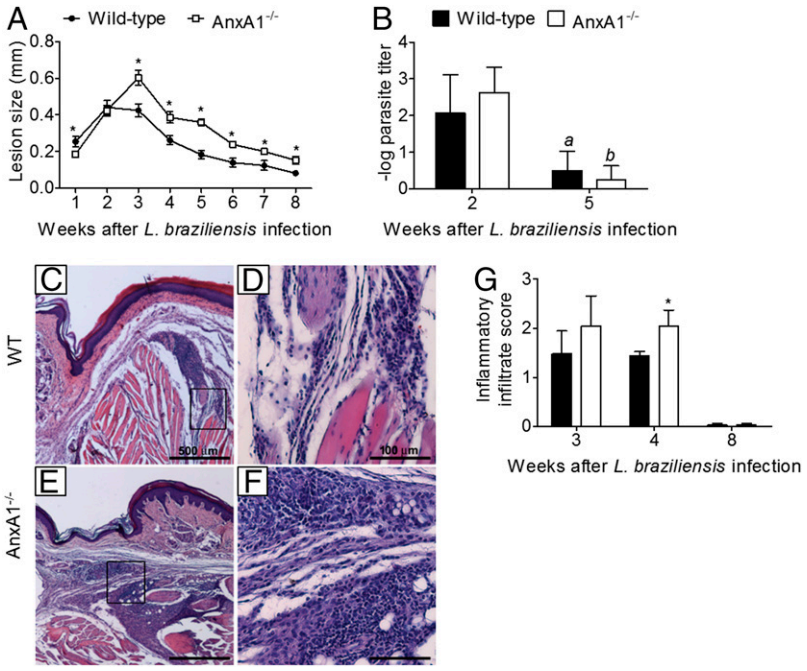

FIGURE 3. Kinetic evaluation of lesion size, parasitism, and histopathological changes during *L. braziliensis* infection in WT and AnxA1$^{-/-}$ mice. WT BALB/c or AnxA1$^{-/-}$ mice were infected in the left hind footpad with 1×10^7 promastigotes of *L. braziliensis*. (**A**) Lesion sizes were monitored weekly with a digital caliper. Data are presented as mean ± SEM. *$p < 0.05$ between WT and AnxA1$^{-/-}$ mice. (**B**) Parasite loads were evaluated at different times after infection. Data are presented as mean ± SD. Data are representative of two experiments with similar results, with at least four mice per group in each time point. Statistically significant differences were assumed when $p < 0.05$ in relation to (*a*) 2 wk postinfection (WT group) and (*b*) 2 wk postinfection (AnxA1$^{-/-}$ group). Histopathological analyses (H&E) were performed 4 wk postinfection for WT (**C** and **D**) and AnxA1$^{-/-}$ (**E** and **F**) mice. Original magnification, ×40 (C and E), ×200 (D and F). (**G**) Quantification of inflammatory infiltrate in mice footpad at 3, 4, and 8 wk postinfection. Data are presented as mean ± SD. Data are representative of two experiments, with similar results, with at least four mice per group in each time point. *$p < 0.05$ between WT and AnxA1$^{-/-}$ mice.

those mice may well compensate for the lack of AnxA1-mediated mechanisms, controlling inflammation and its resolution, and consequently the edema in the infected footpad.

Proinflammatory signaling pathways are upregulated in AnxA1$^{-/-}$ mice

Given the delayed resolution of the inflammatory responses in AnxA1$^{-/-}$ mice, activation of the signaling pathways related to production of inflammatory mediators, which included the MAPK ERK1/2 and NF-κBp65, and the cleavage of caspase-3, as an apoptosis marker, was also investigated. Absence of AnxA1 expression in AnxA1$^{-/-}$ mice was also confirmed in this analysis (Fig. 5A, 5B). As shown in Fig. 5, comparable levels of p-ERK1/2 and p-p65 were detected in WT and AnxA1$^{-/-}$ mice at 2 wk after *L. braziliensis* infection. However, significantly higher activation of these proteins was observed in AnxA1$^{-/-}$ mice at 8 wk, as compared with WT mice, suggesting that the delayed resolution of inflammatory response observed in knockout mice after the second week of infection was associated with more intense and persistent activation of p-ERK1/2 and NF-κB (Fig. 5A, 5C, 5D). Cleaved caspase-3 levels were not altered in AnxA1$^{-/-}$ mice, as compared with WT mice (Fig. 5A, 5E), suggesting that apoptotic mechanisms are activated in *L. braziliensis* infection, regardless of the lack of AnxA1 expression in the knockout mice.

Considering that AnxA1 reduces the expression of iNOS in strains of murine Mϕs (41), as well as the importance of NO for the control of infection by *L. braziliensis* (42), we compared the iNOS expression in tissues of AnxA1$^{-/-}$ and WT infected mice (Fig. 6). Accordingly, AnxA1$^{-/-}$ animals showed an increased expression of iNOS, both at the second (0.71 ± 0.07 versus 0.42 ± 0.06 AU for WT animals) and eighth week postinfection (0.63 ± 0.11 versus 0.0.31 ± 0.05 AU for WT animals). Therefore, our data suggest that the lack of AnxA1 during *L. braziliensis* infection of BALB/c mice results in increased edema and activation of the inflammatory response, including increased IFN-γ levels and iNOS production, leading to efficient control of parasite replication (Fig. 3B).

AnxA1 systemic levels are increased in serum from patients presenting ML

The results described in the present study, in *L. braziliensis*–infected mice, strongly suggest that AnxA1 may be relevant for control and resolution of inflammation during human *L. braziliensis* infection. Therefore, we have compared AnxA1 levels in sera of patients with LCL and ML caused by *L. braziliensis*, as well as in control healthy subjects, paired by sex and age. The

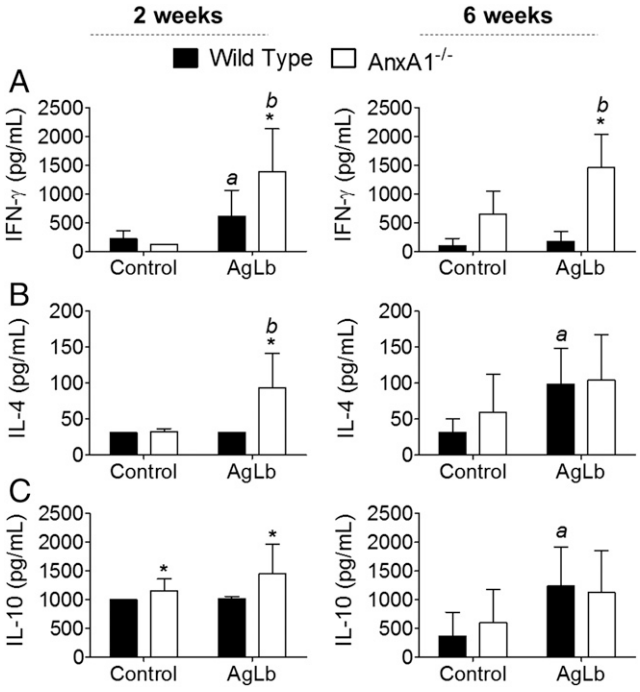

FIGURE 4. Cytokine levels in lymph nodes from WT and AnxA1$^{-/-}$ mice 2 and 6 wk after *L. braziliensis* infection. WT BALB/c or AnxA1$^{-/-}$ mice were infected in the left hind footpad with 1×10^7 promastigotes of *L. braziliensis*. At 2 (left panels) or 6 (right panels) wk postinfection, lymph node cells were stimulated in vitro with AgLb. After 48 h, supernatants were evaluated for (**A**) IFN-γ, (**B**) IL-4, and (**C**) IL-10 levels by ELISA. All data are presented as mean ± SD. Data are representative of two experiments, with similar results, with at least four mice per group. Statistically significant differences were assumed when $p < 0.05$ in relation to (*a*) WT control group and (*b*) AnxA1$^{-/-}$ group. *$p < 0.05$ between WT and AnxA1$^{-/-}$ mice.

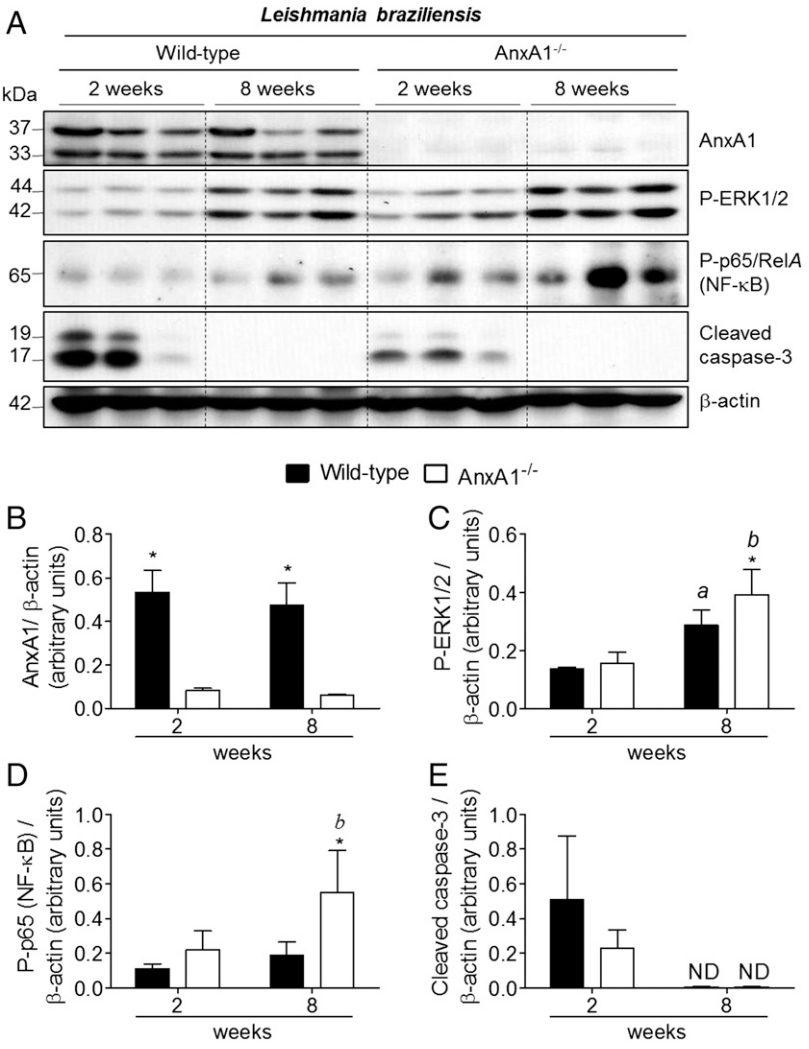

FIGURE 5. Activation status of signaling proteins involved in inflammation and apoptotic pathways after *L. braziliensis* infection in WT and AnxA1$^{-/-}$ mice. WT BALB/c or AnxA1$^{-/-}$ mice were infected in the left hind footpad with 1×10^7 promastigotes of *L. braziliensis*. At 2 and 8 wk postinfection, skin samples from infected footpads were processed and evaluated by Western blotting for signaling proteins from inflammatory and apoptotic pathways. (**A**) Western blotting analysis of p-ERK1/2, p-p65/RelA (NF-κB), and cleaved caspase-3 at 2 and 8 wk postinfection. AnxA1 deficiency was also confirmed by Western blotting. For loading control, membranes were reprobed with β-actin. Densitometric analysis of AnxA1 (**B**), p-ERK1/2 (**C**), p-p65 (NF-κB) (**D**), and cleaved caspase-3 (**E**) bands normalized to β-actin is shown. Data are representative of two experiments, with similar results. All data are presented as mean ± SD of three mice per group in each time point. Statistically significant differences were assumed when $p < 0.05$ in relation to (*a*) 2 wk postinfection (WT group) and (*b*) 2 wk postinfection (AnxA1$^{-/-}$ group). *$p < 0.05$ between WT and AnxA1$^{-/-}$ mice.

characteristics of patients and control subjects are described in Table I. As seen in Fig. 7, the levels of AnxA1 were similar in sera of LCL patients and control subjects. In contrast, significantly increased AnxA1 levels were detected in sera of ML patients, as compared with both LCL patients and control subjects. Therefore, systemic levels of AnxA1 are increased in ML patients, which are known to display chronic exacerbated inflammatory responses.

Discussion

AnxA1 expression has been extensively investigated in models of sterile inflammation, leading to the recognition of its role as a key modulator of both of the innate and adaptive immune systems (15, 43). However, in the context of intracellular parasite infection only few studies (28, 44, 45) have been reported, including the demonstration that AnxA1 expression is locally increased in LCL lesions caused by *L. braziliensis* infection (29). Given that *L. braziliensis* infection may lead patients to develop an exacerbated inflammatory response, with intense tissue damage in chronic disease stages (3, 4, 6), the understanding of the mechanisms involved in the modulation of inflammatory responses, including AnxA1 expression, in leishmaniasis may provide new insights for the development of effective therapies.

BALB/c mice are extremely susceptible to various *Leishmania* species but are resistant to *L. braziliensis* infection (9). Therefore, this model of naturally resolving infection is suitable to evaluate whether AnxA1 expression is modulated during the course of *Leishmania* infection. Indeed, AnxA1 was actively expressed in vitro, in BMMϕs, and in vivo, in BALB/c mice, during *L. braziliensis* infection. Moreover, in these animals, AnxA1 expression was upregulated during the time of increased footpad edema and parasite multiplication, and it was lately downregulated accordingly to control of inflammatory responses and elimination of parasites. Thus, our kinetics experiments suggested an active role of AnxA1 on modulation of the inflammatory responses during *L. braziliensis* infection in BALB/c mice. In contrast, in a self-resolving model of LPS-induced pleurisy, the accumulation of the intact form of AnxA1 (37 kDa) was lost concomitantly to intense neutrophilic infiltration and it was regained at the time points of resolution (25, 46). In agreement, in this model of neutrophilic inflammation, the use of protease inhibitors impaired AnxA1 cleavage and promoted resolution of inflammation (47).

There is significant evidence of reduced phagocytic capacity of Mϕs derived from AnxA1$^{-/-}$ animals, although Mϕs from AnxA1-deficient mice phagocyte opsonized particles similarly to control Mϕs (39, 40, 48). Also, AnxA1 is also known to inhibit other Mϕ functions, including cytokine expression and the production of TNF-α (49). In agreement, our data support the suggestion that BMMϕs from AnxA1$^{-/-}$ are less competent to phagocyte *L. braziliensis* promastigotes. However, after 24 h of infection, AnxA1$^{-/-}$ BMMϕs showed comparable numbers of parasites per infected cells to WT BMMϕs, suggesting that

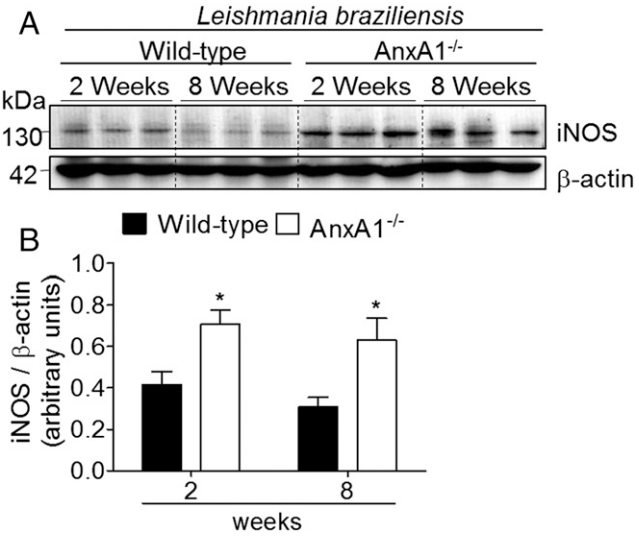

FIGURE 6. iNOS expression after *L. braziliensis* infection in WT and AnxA$^{-/-}$ mice. WT BALB/c or AnxA1$^{-/-}$ mice were infected in the left hind footpad with 1×10^7 promastigotes of *L. braziliensis*. At 2 and 8 wk postinfection, skin samples from infected footpads were processed and evaluated by Western blotting for iNOS expression. (**A**) Western blotting analysis of iNOS at 2 and 8 wk postinfection. For loading control, membranes were reprobed with β-actin. (**B**) Densitometric analysis of iNOS bands normalized to β-actin. Data are representative of two experiments, with similar results. Data are presented as mean ± SD of three mice per group in each time point. *$p < 0.05$ between WT and AnxA1$^{-/-}$ mice.

AnxA1 deficiency did not affect the BMMϕs responses to parasite infection. Interestingly, AnxA1$^{-/-}$ BMMϕs expressed increased levels of TNF-α, after 3 and 24 h, and IL-10 after 24 and 48 h, after *L. braziliensis* infection, indicating that cells were more activated for cytokine production. Therefore, the exacerbated production of TNF-α and IL-10 may ultimately balance the proinflammatory and anti-inflammatory responses, compensating for the lack of AnxA1 expression.

In vivo, the control of *L. braziliensis* replication in AnxA1$^{-/-}$ mice was as effective as in WT mice, and it occurred at the same time points, supporting the hypothesis that AnxA1$^{-/-}$ mice can efficiently control parasite multiplication. These findings differ from those reported for *M. tuberculosis* infection in AnxA1$^{-/-}$ mice, which showed that AnxA1-deficient animals were more susceptible to infection, displaying increased pulmonary bacterial burden and exacerbated and disorganized granulomatous inflammation (30, 39). These pathological manifestations correlated with an impaired ability of AnxA1$^{-/-}$ dendritic cells to activate naive T cells, leading, consequently to low IFN-γ production. In our study, lack of AnxA1 protracted the resolution of inflammatory responses, but it did not impact the course of disease or activation of specific T cell responses. Assessment of cytokine production by popliteal draining lymph node cells revealed increased and sustained parasite-specific IFN-γ levels. Thus, deficiency of AnxA1 in the model of *L. braziliensis* BALB/c mice infection did not compromise the activation of IFN-γ–producing cells and other specific cellular immune responses, resulting, in turn, in an effective capacity to eliminate parasites.

AnxA1 was demonstrated to modulate Ag-specific CD4$^+$ T cell proliferation and cytokine production (26). AnxA1 also induces IL-10 production (41, 50, 51). IL-4 and IL-10, in addition to the proresolving mediators, are important in the regulation of IFN-γ production by inhibiting exacerbated Th1 responses and controlling inflammatory responses (52). Although IFN-γ has an outstanding importance in *Leishmania* spp. infection, exacerbated type 1 cellular responses are associated with the pathogenesis of leishmaniasis (53), which could be due to defects in the activation of proresolving pathways, including those induced by AnxA1. However, increased levels of IL-10 were also observed in *L. braziliensis*–infected AnxA1$^{-/-}$ mice. In this case, other stimuli to IL-10 production may be acting, including lipoxin A_4, which may compensate for the lack of AnxA1 in this model (21). Similar compensatory mechanisms have been demonstrated in a genetic-deficient background of the glucocorticoid-induced protein GILZ (46).

The increased IL-4 and IL-10 levels observed after *L. braziliensis* infection may have therefore contributed to recovery and maintenance of tissue homeostasis. Importantly, they did not impair parasite control. However, the increased levels of anti-inflammatory cytokines may have resulted in a delayed ability of AnxA1-deficient animals to resolve inflammation. One possible explanation for the delayed resolution of inflammatory responses in AnxA1 during *L. braziliensis* infection may be the exacerbated activation of signaling pathways related to production of proinflammatory mediators in late periods of infection, such as the MAPK ERK1/2 and NF-κB pathways. In agreement, the increased expression of ERK1/2 observed at 8 wk postinfection in AnxA1$^{-/-}$ mice may be related to an extended survival of inflammatory cells at the site of infection, similarly as observed in a model of Chagas' disease in which the activation of ERK1/2 correlated with inflammatory cell survival (54). Recently, a delay in neutrophil apoptosis in mice infected by *L. major* was demonstrated, an effect mediated by the activation of ERK1/2 (55). Alternatively, the ERK1/2 activation at this late period of infection may have affected the increased production of the anti-inflammatory cytokine IL-10 as a feedback, compensatory mechanism for regulation of tissue inflammation (56).

The activation of NF-κB has a major impact in *Leishmania* spp. infection due to its participation in the production of IL-12, and thus the development of Th1 responses (57, 58). Moreover, manipulation of the NF-κB pathway is strategically used by *Leishmania* spp. to subvert the host immune response in susceptible mice models (59–65). Therefore, the increased levels of p65 NF-κB correlated with the delay in the edema resolution and inflammatory responses during the late stages of *L. braziliensis* infection in AnxA1$^{-/-}$ mice. Finally, caspase-3 activation was not affected by the lack of AnxA1. The high caspase-3 cleavage (and consequent activation) in the second week postinfection may be related to increased apoptosis of inflammatory cells in the microenvironment of the lesion in both mice (25).

Table I. Characteristics of LCL and ML patients and control subjects

Groups	LCL			ML		
	Control	Patient	*p* Value	Control	Patient	*p* Value
Number of individuals	10	10	NS	12	12	NS
Average age, y (range)	22.6 (9–35)	21.7 (9–33)	NS	42.8 (25–76)	45.4 (29–74)	NS
Male, *n* (%)	5 (50.0)	5 (50.0)	NS	10 (83.3)	10 (83.3)	NS

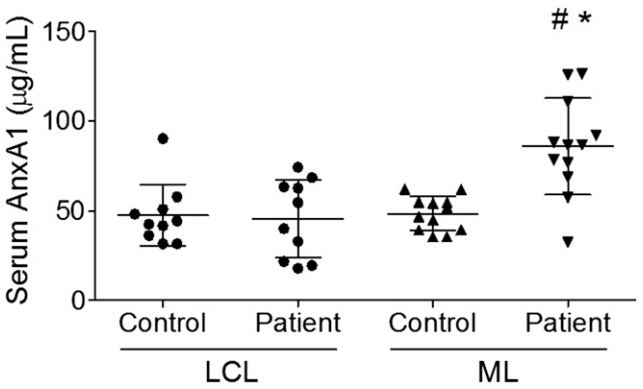

FIGURE 7. Serum levels of AnxA1 in LCL and ML patients and control subjects. AnxA1 levels were measured in serum samples from patients with LCL or ML, as well as in paired control subjects. All data are presented as mean ± SD of at least 10 patients per group. $^{\#}p < 0.05$ in relation to the LCL patients group, $^{*}p < 0.05$ between ML control subjects and ML patients.

Exogenous administration of AnxA1 protein reduced the iNOS message and therefore the NO production in Mϕs stimulated with LPS (41). Treatment with dexamethasone also reduced the expression of iNOS in response to LPS, through an AnxA1-dependent mechanism (66). In the same vein, LPS-stimulated Mϕs from AnxA1$^{-/-}$ produced higher levels of iNOS as compared with WT Mϕs (40). In agreement, AnxA1$^{-/-}$ mice displayed higher iNOS expression following *L. braziliensis* infection. This finding suggests that AnxA1 may modulate the levels of iNOS and consequent NO production during *L. braziliensis* infection. As *L. braziliensis* is highly susceptible to NO (42), the major microbicidal mechanism during infection, parasite control was effectively achieved in the absence of AnxA1, regardless of the higher levels of IL-10 and IL-4.

Finally, serum AnxA1 levels were significantly increased in ML patients, but not in patients presenting with LCL. A recent study has shown increased local expression of AnxA1 in lesions of Brazilian patients with LCL caused by *L. braziliensis* (29). These contrasting findings may be related to the stage of infection in the different groups of patients. Whereas LCL lesions develop on average 3 mo after infection and are more promptly diagnosed, ML is a systemic disease that develops years after a primary LCL lesion, as a late complication and chronic inflammatory condition due to *L. braziliensis* infection. Although further studies are required, it is reasonable to presume that the expression of AnxA1 has been detected only locally in LCL lesions (29) owing to moderate inflammatory responses, which may eventually spontaneously heal. Alternatively, in a systemic infection characterized by a chronic and more severe inflammation, AnxA1 may become detectable in sera of ML patients.

Persistence of parasites concomitantly to an intense inflammatory response is still a puzzling question in human ML. More intriguingly, in ML patients, high IFN-γ/IL-10 secretion profiles in response to *Leishmania* Ags are present (67). Interestingly, in patients infected with *Plasmodium vivax*, AnxA1 expression was increased in regulatory T cells and decreased in CD4^{+} and CD8^{+} T cells. Additionally, it has been suggested that AnxA1 may contribute to IL-10 production in plasma of patients with vivax malaria (45). Considering the increase in AnxA1 serum levels in ML patients, our findings suggest that upregulation of AnxA1, similarly to other anti-inflammatory markers (68), is not effective to counterbalance the higher levels of proinflammatory mediators present in ML patients, including higher levels of TNF-α. However, a deficiency in the engagement of AnxA1 to its receptor or disturbances in downstream pathways may be determinant for maintenance of the chronic inflammatory condition. Curiously, increased expression of AnxA1 was shown in biopsies of patients under anti-TNF therapy for ulcerative colitis and in mice lacking TNFR1, indicating that blocking of TNF signaling may enhance AnxA1 bioactitity (69). In agreement, a deficit in the expression of IL-10R has been reported in ML patients (68), accounting for unbalanced type 1 and 2 immune responses in ML (68).

The presence of AnxA1, at the onset of inflammation, is important to temper inflammation by inhibiting several proinflammatory mediators that are produced, such as PGs, cytokines, chemokines, and adhesion molecules, decreasing leukocyte infiltration into tissue. In agreement, it has been shown that the absence of AnxA1 is associated with development of more severe inflammation in different models of inflammatory diseases (31, 70–73), suggesting that when this counterregulatory pathway is defective, the inflammation is overshot. Particularly in our experimental settings, lesions are bigger in *Leishmania*-infected AnxA1$^{-/-}$, with more intense inflammatory cell infiltrate, cytokine production by lymph nodes cells, and expression of inflammatory-associated pathways, as compared with WT mice. From a translational perspective, the higher levels of AnxA1 in patients with ML, as compared with those presenting with LCL, indicate that the upstream branch of the AnxA1 pathway is functional and upregulated to control/attenuate the exacerbated inflammatory response in a more severe and chronic inflammatory milieu as compared that present in LCL patients. In the context of other chronic inflammatory human diseases, a similar pattern, that is, increased AnxA1 levels, has been found in pre-eclamptic women (74), Alzheimer disease (75), and ulcerative colitis (76).

In conclusion, our data indicate that AnxA1 is actively expressed and may be required to control tissue inflammation in *L. braziliensis* infection. Therefore, further investigations on the role of AnxA1 and its downstream pathway may eventually provide insights to new therapeutic strategies to better manage tegumentary leishmaniasis patients.

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Disclosures

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