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Expression of annexin A1 in *Leishmania*-infected skin and its correlation with histopathological features

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ABSTRACT

Introduction: The aim of this study was quantify annexin A1 expression in macrophages and cluster of differentiation 4 (CD4)+ and cluster of differentiation 8 (CD8)+ T cells from the skin of patients with cutaneous leishmaniasis (n=55) and correlate with histopathological aspects. **Methods:** Infecting species were identified by polymerase chain reaction-restriction fragment length polymorphism, and expression of annexin A1 was analyzed by immunofluorescence. **Results:** All patients (n = 55) were infected with *Leishmania braziliensis*. Annexin A1 was expressed more abundantly in $CD163^+$ macrophages in infected skin ($p < 0.0001$) than in uninfected skin. In addition, macrophages in necrotic exudative reaction lesions expressed annexin A1 at higher levels than those observed in granulomatous ($p < 0.01$) and cellular lesions $p < 0.05$). This difference might be due to the need to clear both parasites and necrotic tissue from necrotic lesions. $CD4^+$ cells in cellular lesions expressed annexin A1 more abundantly than did those in necrotic ($p < 0.05$) and granulomatous lesions ($p < 0.01$). Expression in $CD8^+$ T cells followed the same trend. These differences might be due to the pervasiveness of lymphohistiocytic and plasmacytic infiltrate in cellular lesions. **Conclusions:** Annexin A1 is differentially expressed in $CD163^+$ macrophages and T cells depending on the histopathological features of *Leishmania*-infected skin, which might affect cell activation.

Keywords: Cutaneous leishmaniasis. Annexin A1. Lymphocytes. Macrophages.

INTRODUCTION

American cutaneous leishmaniasis is a non-contagious infection of the skin and mucosa by parasitic protozoans of the genus *Leishmania*[1] [2] [3]. It is transmitted through female *Phlebotomus* and *Lutzomyia* sand flies[4]. The infection is diagnosed based on a compendium of clinical, epidemiological, and laboratory characteristics[2].

The host immunological response determines to a significant extent whether the infection persists or is cleared has high relevance for determining cure or persistence[5] [6]. In early infection, no macroscopic pathological changes occur in the epidermis[7]. Following initial acute inflammation, the infection progresses to a silent phase lasting a few weeks to months, during which the parasite proliferates without clinical manifestation. The silent phase ends with extensive inflammation and lesion formation at the infection site[8]. Lesion healing and parasite clearance correlate with a preponderance of chronic rather than acute inflammatory cells in the infected tissue[9]. However, while a T cell-mediated response is essential to clear parasites in most cases[10], the dogma *Th1 = good/Th2 = bad* is somewhat inadequate[11]. For instance, hyperinflammatory collateral damage from T helper type 1 (Th1) response has been reported, along with a variable T helper type 2 (Th2) response dependent on cytokine release and other T cells[12] [13]. Moreover, Cardoso et al.[14] demonstrated that cluster of differentiation 8 **(**$CD8)^+$ T cells in patients with subclinical *Leishmania braziliensis* infection secrete interferon gamma (IFN-γ) to activate macrophages and facilitate parasite clearance. Notably, Pereira-Carvalho et al.[15] showed that T cells maintain activation levels at approximately 2 years after the end of therapy, and lymphocytes from well-healed lesions recognize leishmanial stimuli and proliferate upon exposure. Taken together, the data indicate that the immune response against *Leishmania* is very complex, and it is essential to understand better the processes at the infection site and the molecules present at inflammation site. Annexin A1 (ANXA1), also known as lipocortin-1, is a 37-kDa calcium- and phospholipid-binding protein involved in several biological processes, including suppression of inflammation[16] [17] [18]. Indeed, ANXA1 modulates leukocyte extravasation to the site of inflammation and regulates cytokine release in acute, chronic, or systemic inflammation[19] [20].

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Its role in adaptive immunity is poorly understood; however, it has been shown to inhibit proliferation and differentiation of T cells by modulating T cell receptor signaling[18]. To help define the role of ANXA1 in adaptive immunity, we analyzed its expression in $CD163^+$ macrophages, $CD4^+$, and $CD8^+$ T cells from skin biopsies of patients with cutaneous leishmaniasis, and investigated its correlation with histopathological features. This study is important for shows the relevance of ANXA1 dynamics in the immune system regulation during cutaneous leishmaniasis.

METHODS

Patients

This cross-sectional study was performed in 55 patients with symptomatic cutaneous leishmaniasis who were treated at Julio Müller University Hospital [*Hospital Universitário Julio Müller* (HUJM)], Cuiabá, State of Mato Grosso, Brazil, between February 2012 and November 2013. Patients were of both sexes, aged 18-80 years. Patients who presented other infectious or immunosuppressive diseases, as well as those who were already being treated for leishmaniasis were excluded. Twenty patients undergoing surgery for colorectal cancer were used as control, and skin biopsies were collected at the incision site.

The Ambulatory of American Tegumentary Leishmaniasis/ HUJM is used in the State of Mato Grosso to diagnose and treat leishmaniasis. To confirm infection, patients received clinical examination, as well as histopathological and parasitological tests suggestive of *Leishmania* infection, including blades from aspirate, shave, and smear of injured tissue, as well as cultures from aspirate and biopsy of the lesion. Following diagnosis, infecting species were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Histopathology

Patient received local anesthesia and lesions were previously disinfected. Tissue samples were obtained by biopsy with a 4-mm punch. Tissues were then immersed in 10% formol, dehydrated through a gradient of crescent alcohol, clarified in xylene, and embedded in paraffin. Samples were sectioned at 3μm by using a HYRAX M60 microtome (Carl Zeiss, Germany), deparaffinized in xylene, hydrated in decreasing concentrations of alcohol, and stained with hematoxylin-eosin. Lesions were then scored by pathologists who were blinded to the study according to the criteria defined by Magalhães[21] for cellular exudative reaction (CER), granulomatous exudative reaction (GER), necrotic exudative reaction (NER), granulomatous-necrotic exudative reaction (GNER), and tuberculoid exudative reaction (TER).

ANXA1 expression

Histological sections were fixed on slides with a biological adhesive, deparaffinized with xylene, hydrated in decreasing concentrations of alcohol, and incubated for 1h in 0.21% sodium citrate pH 6.0 set at 70°C. Samples were then treated for 30 min with 3% hydrogen peroxide in 70% methanol to block endogenous peroxidase, permeabilized in 0.4% Tween 20 in phosphate buffer saline for 15 min, and blocked for 30 min with 5% bovine serum albumin (Sigma-Aldrich, Rio de Janeiro, Brazil) in phosphate buffered saline (PBS). Sections were then probed with rabbit anti-ANXA1 (Invitrogen, USA; 1:200), and labeled for 1h at 25°C in a humidified chamber with secondary goat anti-rabbit immunoglobulin G (IgG) conjugated to Alexa Fluor 488 (Invitrogen, Eugene, OR, USA; 1:50). Antibodies were diluted in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Sections were also stained with the nuclear fluorescent dye 4′,6-diamidino-2-phenylindole (Sigma, USA) to facilitate morphological characterization. Finally, slides were washed in PBS, mounted in 1:1 glycerin: PBS, and examined under an AxioScope.A1 microscope (Carl Zeiss, Germany).

We quantified ANXA1 expression in AxioVision (v.4.8.1, 2009), using median optical density in arbitrary units (AU) (scale values: 0 to 255). For densitometry, images were obtained with a 20× objective lens, and readings are reported as mean ± SEM. ANXA1 expression was analyzed in the cytoplasm of $CD163^+$ macrophages, $CD4^+$, and $CD8^+$ T cells (n = 10 cells types per each patient).

Identification of $CD163^+$ monocytes, $CD4^+$ and $CD8^+$ T cells and *Leishmania* parasites

To identify host and parasite cells, sections were probed for 18h at 4°C in a humidified chamber with mouse primary antibodies against CD4 (Invitrogen, USA; clone RPA-T4, 1:100), CD8 (BD Biosciences, USA; clone RPA-T8, 1:100), CD163 (Cell Marque, USA; clone EP152, 1:200), and *Leishmania* (Invitrogen, USA; 1:300). Subsequently, samples were labeled for 1h at 25°C in a humidified chamber with secondary goat anti-mouse antibody conjugated to Alexa Fluor 546 (Invitrogen, Eugene, OR, USA; 1:50). All antibodies were diluted in 1 % BSA in PBS. Sections were also stained with the nuclear fluorescent dye 4′,6-diamidino-2-phenylindole (Sigma, USA) as described. Host and parasite cells were identified using morphometric tools in AxioVision (v.4.8.1, 2009).

DNA extraction from skin biopsies

Deoxyribonucleic acid (DNA) was extracted using Wizard™ Genomic DNA Purification kit (Promega, WI, USA) from skin biopsies frozen and stored at -80°C. DNA was quantified using NanoDrop®.

Identification of infecting *Leishmania* species

Internal transcribed spacer 1 (ITS1) ribosomal deoxyribonucleic acid (rDNA)[22] was amplified in 50-μL PCR reactions containing 4μL DNA extract, 200μM dNTPs, 1.5mM $MgCl_2$, 1U GoTaq® DNA polymerase (Promega, USA), and 20 pmol each of forward primer with sequence 5'-ctg gat cat ttt ccg atg-3' and reverse primer with sequence 5'-tga tac cac tta tcg cac tt-3'. Reactions were denatured at 94 ºC for 5 min, and amplified over 30 cycles at 94ºC for 30 s, 61ºC for 1 min, and 72ºC for 1 min, followed by final extension at 72ºC for 10 min. Amplification products (11μL) were digested at 65 °C for 20 min with 1μL Sau3AI in 5μL cutSmart buffer and 33μL distilled water. Restriction products were electrophoresed and visualized by silver staining using a GenePhor electrophoresis unit (GE Healthcare) and a high-resolution 12.5% polyacrylamide gel

kit (GeneGel Excel 12.5/24 kit, GE Healthcare). Reference species and reactions without template DNA were used as controls, in accordance with World Health Organization (WHO) standards. The reference species *L. braziliensis* (MHOM/BR/1975/M2903/IOC/L566), *L. amazonenses* (IFLA/BR/1967/PH8/IOC/L575), *L. lainsoni* (MHOM/BR/1981/M6426/IOC/L1023), *L. naiffi* (MDAS/BR/1979/M553/IOC/L1365), and *L. shawi* (MCEB/BR/1984/M8408/IOC/L1545) were kindly provided by the *Leishmania* Collection of the Instituto Oswaldo Cruz, *Instituto Oswaldo Cruz*/*Fundação Oswaldo Cruz* (IOC/FIOCRUZ).

Statistical analyses

We compared ANXA1 expression using one-way analysis of variance (ANOVA) and Bonferroni's post-hoc test in GraphPad Prism v. 5.01 for Windows (La Jolla, CA, USA); Values were considered significant and displayed as symbols in the figures as: one symbol, p value below 0.05; two symbols, p value below 0.01; three symbols, p value below 0.001.

Ethical considerations

Participation was voluntary, and patients signed informed consent forms of their own cognizance. This study was approved by the Research Ethical Committee of Julio Müller University Hospital (625-CEP-HUJM/2009).

RESULTS

Identification of infecting *Leishmania* species

The infecting species in all 55 cases was identified by PCR-RFLP to be *Leishmania braziliensis* (**Figure 1**).

Histopathological analysis

Nine (16.3%) patients were women, and 46 (83.7 %) were men. Patients were 18-72 years old, with mean age 38 years. All patients presented skin lesions, which were scored cellular (n = 25; 45.5%), necrotic (n = 9; 16.3%), and granulomatous exudative reactions (n = 21; 38.2%). Tissue samples tested positive for *Leishmania* by immunofluorescence (**Figure 2**).

ANXA1 expression

Expression of ANXA1 in macrophages and T cells recruited to leishmaniasis lesions was measured by immunofluorescence (**Figure 3** and **Figure 4**). $CD163^+$ macrophages expressed ANXA1 at basal levels in uninfected individuals. However, $CD163^+$ macrophages in infected lesions expressed ANXA1 more abundantly (107.0 ± 2.7AU versus 64.6 ± 3.0AU in uninfected control, $p < 0.0001$) (**Figures 3A-H**), even though expression did not correlate with age of the lesion ($r^2 = 0.0031$, **Figure 3I**). Expression was higher in necrotic lesions (123.5 ± 6.9AU) than in granulomatous (100.0 ± 4.1AU, $p < 0.01$) and cellular lesions (104.6 ± 3.0AU, $p < 0.05$) (**Figure 3H**). Expression did not correlate as well with time of lesion, with r^2 0.0462, 0.2403, and 0.1735 for granulomatous, cellular, and necrotic lesions, respectively. In all cases, ANXA1 was expressed in the cytosol and cell membrane (**Figure 3B** and **Figure E**).

ANXA1 was also mainly expressed in the cytoplasm of $CD8^+$ and $CD4^+$ T cells (**Figures 4A-F**). Expression in $CD8^+$ cells was higher in cellular lesions (121.3 ± 9.0U) than in granulomatous (76.0 ± 11.4AU, $p < 0.05$) and necrotic lesions (77.0 ± 10.4AU, $p < 0.05$). Similarly, expression in $CD4^+$ cells was higher in cellular (123.9 ± 11.6AU) than in granulomatous (87.7 ± 5.2AU, $p < 0.05$) and necrotic lesions (53.7 ± 15.7AU, $p < 0.01$) (**Figure 4G** and **Figure H**). As in $CD163^+$ macrophages, expression in $CD8^+$ and $CD4^+$ T cells did not correlate with age of the lesion, with r^2 0.0267 and 0.00488, respectively (**Figure 4I** and **Figure J**). Accordingly, expression did not correlate with age of specific lesions, with r^2 0.0274, 0.0213, and 0.1011 for $CD8^+$ T cells in granulomatous, cellular, and, necrotic lesions, respectively, and 0.04981, 0.0024, and 0.0859 for $CD4^+$ T cells.

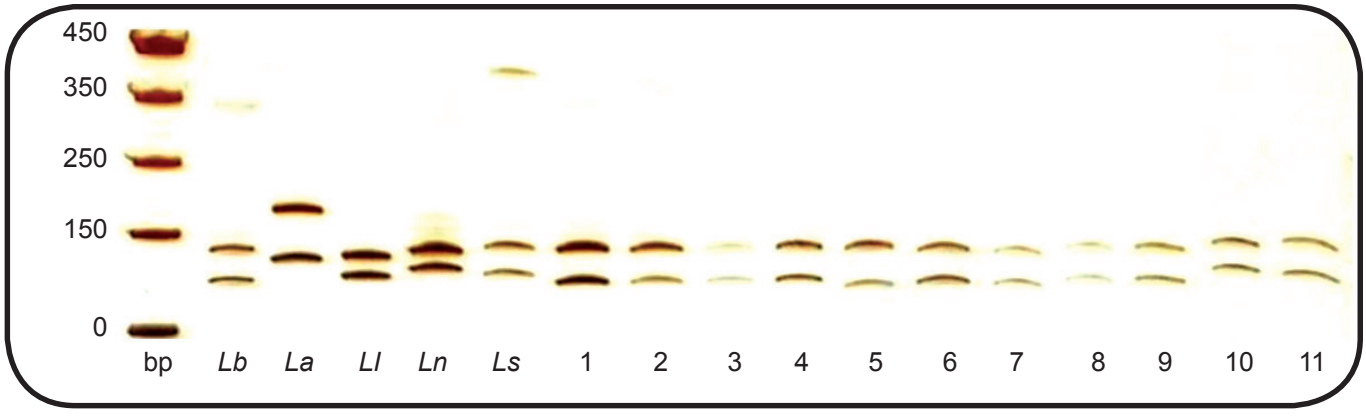

FIGURE 1 - Identification of the infecting *Leishmania* species by PCR-RFLP. Representative silver-stained 12.5% polyacrylamide gel (GenePhor®) of RFLP patterns generated by digesting PCR-amplified ITS1 rDNA with Sau3AI. **bp:** 100-bp molecular weight marker; ***Lb:*** *Leishmania braziliensis;* **La:** *Leishmania amazonensis;* ***Ll****: Leishmania lainsoni;* ***Ln:*** *Leishmania naiffi;* ***Ls:*** *Leishmania shawi*; **numbers 1 to 11:** representative samples from patients with cutaneous leishmaniasis; **PCR-RFLP:** polymerase chain reaction-restriction fragment length polymorphism; **ITS1:** internal transcribed spacer 1; **rDNA:** ribosomal deoxyribonucleic acid.

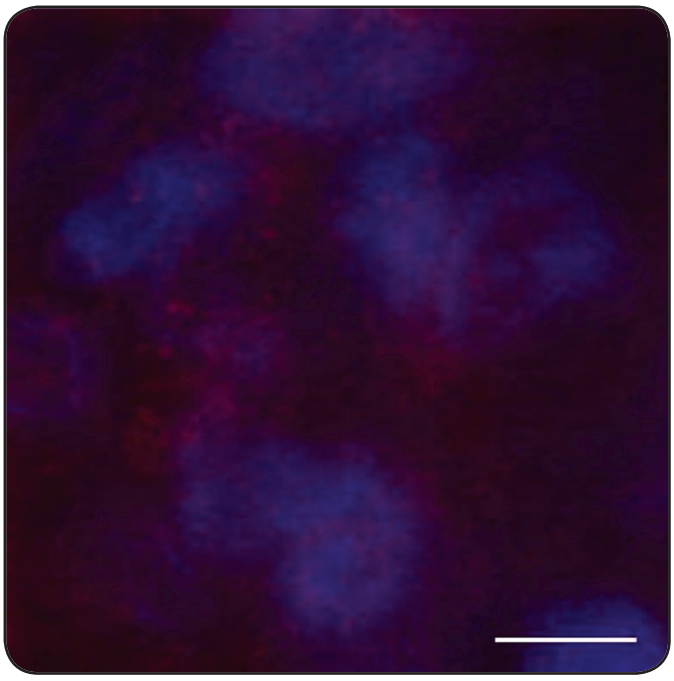

FIGURE 2 - Staining of *Leishmania* parasites in infected skin. A cellular exudative reaction lesion with macrophages and *Leishmania* parasites. Parasites are stained with antibodies conjugated to Alexa Fluor 546 (red), and nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole)(blue). Scale bar: 10µm.

DISCUSSION

In this study, the patients were infected with *L. braziliensis* alone; notably, patients examined by Carvalho et al[4] in HUJM/UFMT, Cuiabá, Brazil were also infected by *L. braziliensis* alone, suggesting that this species is the most prevalent in different areas in Brazil.

ANXA1 has pleiotropic and pluripotent effects in several biological processes, including inflammation and tumorigenesis [19] [20] [23]. However, the role of ANXA1 in adaptive immunity is poorly defined. Published data indicate that ANXA1 expression is lower in lymphocytes than in neutrophils and monocytes[24] [25], and imply that ANXA1 modulates T cell-mediated immune response[26] by activating specific signaling pathways and suppressing transcription factors[26] [27].

The host immune response elicited by *Leishmania* has been widely studied, and was shown to be extremely complex and variable[11] [28]. However, the role of ANXA1 in this response is unknown. Our data demonstrate that skin macrophages from patients with cutaneous leishmaniasis express ANXA1 more abundantly than do macrophages from uninfected skin, major histocompatibility complex those observed in other leukocytes at steady state and during acute[29] or chronic inflammation[30]. Abundant expression at infection sites also suggests that the protein may be involved in phagocytosis of parasites. Indeed, the literature shows that ANXA1 regulates phagocytosis, macrophage differentiation, and activation[20] [31] [32] [33] by inducing the expression of CD40, CD54, CD80, CD84, major histocompatibility complex class II (MHCII), and CCR7[33]. Finally, Collins et al[34] detected ANXA1 in vacuoles containing *L. mexicana*, implying that the protein might facilitate vesicle fusion with endosomes.

The literature also suggests that ANXA1 in phagocytes facilitates clearance of apoptotic cells[32]. Indeed, macrophages in ANXA1-deficient mice have reduced capacity to clear apoptotic bodies[31]. Notably, Tzelepis et al[35] reported that these mice are highly susceptible to *Mycobacterium tuberculosis*, and that ANXA1 expression is significantly downregulated in infected dendritic cells, suggesting that suppression of ANXA1 is a critical mechanism for immune evasion by *Mycobacterium tuberculosis*. In our patients, ANXA1 expression is more abundantly expressed in necrotic lesions than in cellular or granulomatous lesions, implying that expression in necrotic lesions is stimulated by the need to clear both parasites and necrotic tissue. Indeed, several studies have demonstrated that ANXA1 expression can be precisely calibrated depending on the stimulus, of which glucocorticoids[36] and tumor necrosis factor alpha (TNF-α)[18] are the most extensively characterized.

We found ANXA1 to be expressed in $CD4^+$ and $CD8^+$ T cells as well, suggesting that the protein is upregulated during the immunological response to *Leishmania.* T cells in cellular lesions express ANXA1 robustly, presumably due to widespread inflammation, as indicated by accumulation of lymphohistiocytic and plasmacytic infiltrate, edema, and absence of granuloma. ANXA1 has been described in the literature as a key regulator of T cell activation and migration to inflammatory sites[29] [30] [33], and of signaling pathways (p38, ERK MAPK, Akt, and NF-κB) that control production of cytokines such as TNF-α, INF-γ, IL-2, and IL-17[37] [38]. ANXA1 also regulates the differentiation and proliferation of lymphocytes[18]. For instance, ANXA1 was recently demonstrated to regulate the differentiation of Th0 cells into Th1[33], although Th2 cells predominate in ANXA1-deficient animals[37]. Finally, ANXA1 expression did not correlate with the age of the lesion, suggesting that expression might be regulated by pro-inflammatory mediators at inflamed sites, as has been reported[18] [36].

Our study evaluates the differential ANXA1 expression in different histophatological lesions of patients with cutaneous leishmaniasis (CL). The results were very positive; however, all patients were infected with *Leishmania brasiliensis.* It is possible that other parasite species shows a different pattern of expression.

In summary, our data show for the first time that ANXA1 is differentially expressed in macrophages and T cells in lesions due to leishmaniasis, and expression is dependent on the histopathological characteristics of the lesion. We anticipate that future studies will further clarify the regulatory mechanism of cell action of ANXA1 during *Leishmania* infection.

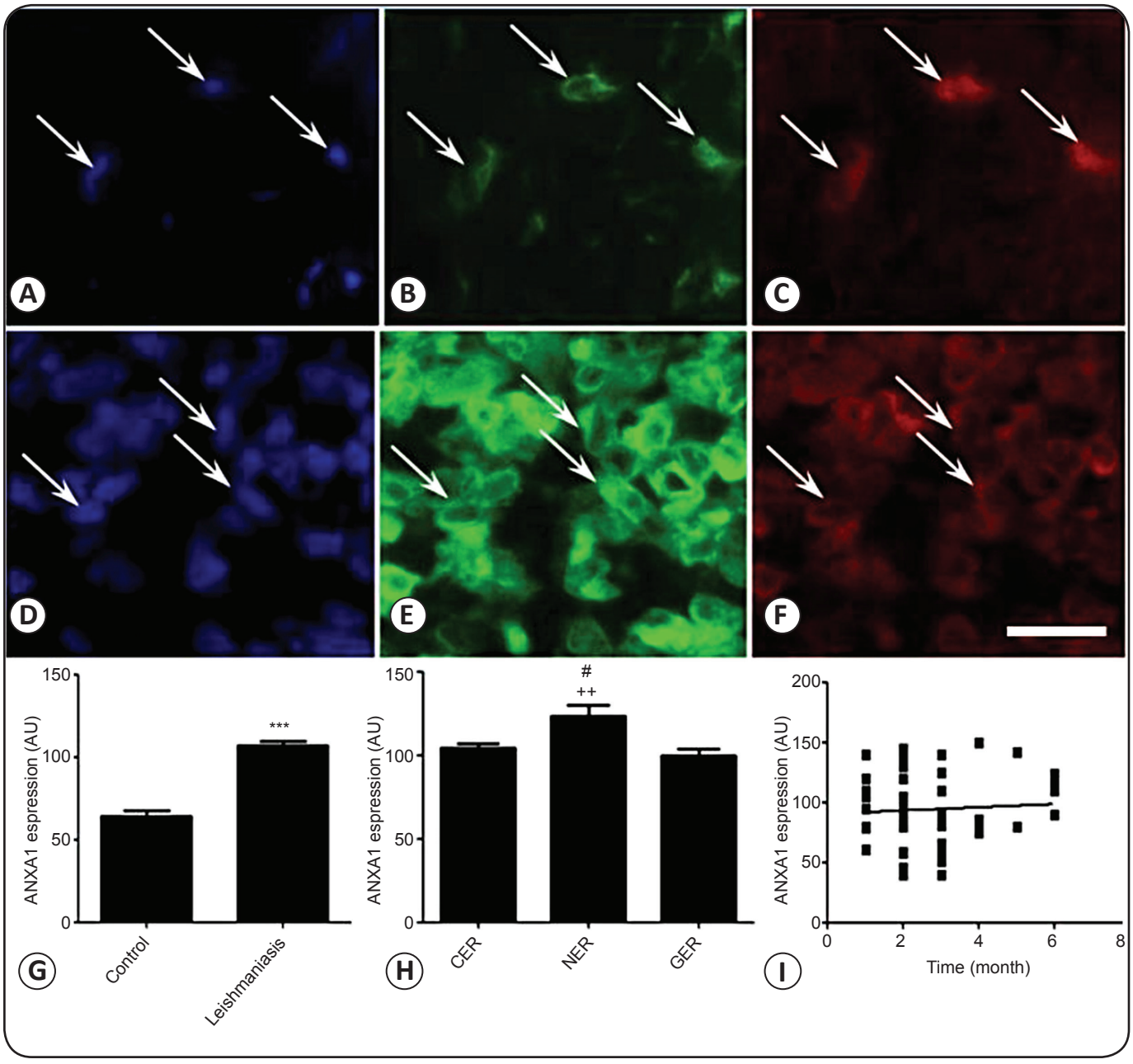

FIGURE 3 - ANXA1 expression in CD163$^+$ macrophages in *Leishmania*-infected skin. A-C: Basal ANXA1 expression in CD163$^+$ macrophages (arrows) in uninfected skin. D-F: Cellular exudative reaction lesion with CD163$^+$ macrophages in lymphohistiocytic and plasmacytic infiltrate. A and D: Nuclei stained with DAPI. B and E: CD163$^+$ macrophages expressing ANXA1 in the cytoplasm. C and F: Immunofluorescent staining of the macrophage marker CD163. G: ANXA1 expression in macrophages in uninfected and infected skin. H: ANXA1 expression in macrophages in necrotic (NER), cellular (CER), and granulomatous (GER) lesions. I: ANXA1 expression in CD163$^+$ macrophages did not correlate with age of the lesion. ANXA1: annexin A1; **CD:** cluster of differentiation; **DAPI:** 4′,6-diamidino-2-phenylindole; **NER:** necrotic exudative reaction; **CER**: cellular exudative reaction: **GER**: granulomatous exudative reaction. **Statistical analysis as described in Methods:** ***p < 0.0001 against uninfected skin. ++p < 0.01 against GER lesions. #p < 0.05 against CER lesions. Scale bar: 50μm.

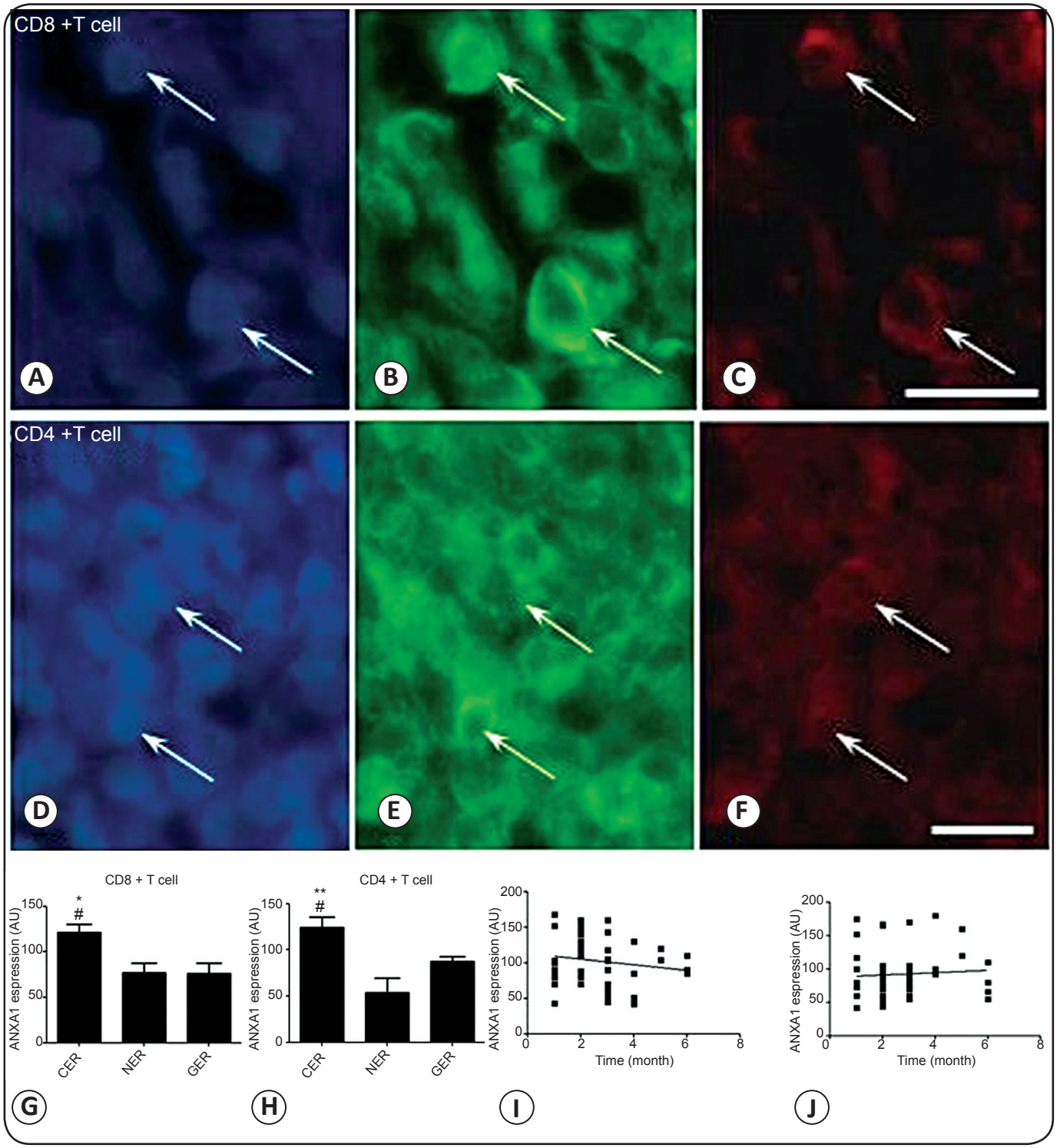

FIGURE 4 - ANXA1 expression in CD4⁺ and CD8⁺ T cells in *Leishmania*-infected skin. ANXA1 expression in (A-C) CD8⁺ and (D-F) CD4⁺ T cells in the lymphohistiocytic and plasmacytic infiltrate of a cellular exudative lesion. A and D: Nuclei stained with DAPI. B and E: T cells expressing ANXA1 in the cytoplasm. C and F: Immunofluorescent staining of T cell markers CD8 and CD4. G and H: ANXA1 expression in cellular (CER), granulomatous (GER), and necrotic (NER) exudative reaction lesions. I and J: ANXA1 expression in CD8⁺ and CD4⁺ T cells did not correlate with age of lesion. ANXA1: annexin A1; **CD4⁺:** cluster of differentiation 4⁺; **CD8⁺:** cluster of differentiation 8⁺; **DAPI:** 4′,6-diamidino-2-phenylindole; **CER**: cellular exudative reaction; **GER**: granulomatous exudative reaction; **NER:** necrotic exudative reaction. **Statistical analysis as described in Methods:** *p < 0.05; **p < 0.01 against NER lesions. #p < 0.05 against CER lesions. Scale bar: 100μm.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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