

Analysis of the Myeloid-Derived Suppressor Cells and Annexin A1 in Multibacillary Leprosy and Reactional Episodes

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Abstract

Background: Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. Patients have distinct clinical forms, and host's immunological response regulate those manifestations. In this work, the presence of the myeloid-derived suppressor cell and the regulatory protein annexin A1 is described in patients with multibacillary leprosy and with type 1 and 2 reactions.

Methods: Patients were submitted to skin biopsy for histopathological analysis to obtain bacilloscopic index. Immunofluorescence was used to detect myeloid-derived suppressor cells and annexin A1.

Results: The data demonstrated that the presence of granulocytic and monocytic myeloid-derived suppressor cells in leprosy patients. The high number of monocytic myeloid-derived suppressor cells were observed in lepromatous leprosy and type 2 reactional patients with Bacillus Calmette–Guérin (BCG) vaccination scar. The presence of annexin A1 was observed in all myeloid-derived suppressor cells. In particular, the monocytic myeloid-derived suppressor cell in the lepromatous patients has higher levels of this protein when compared to the reactional patients. This data suggest that the higher expression of this protein may be related to regulatory response against a severe infection, contributing to anergic response. In type 1 reactional patients, the expression of annexin A1 was reduced.

Conclusions: Myeloid-derived suppressor cell are present in leprosy patients and annexin A1 might be regulated the host response against *Mycobacterium leprae*.

1. Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. Transmission occurs by very close and prolonged coexistence with non-treated multibacillary leprosy patients [1]. Patients have distinct clinical forms, affecting mainly the skin and the nerves, causing lesions resulting from inflammatory processes [2, 3]. The lesion extension may be related to the genetic background and immune response [4]. Some individuals, in the beginning, during or after the end of the treatment, may present acute clinical manifestations due to release of antigens and hypersensitivity reactions, known as leprosy reactions: type 1 reaction (T1R) or reverse reaction, and type 2 reaction (T2R) or erythema nodosum leprosum (ENL) [4, 5].

In the literature, some studies have reported some immunoregulatory cells called myeloid-derived suppressor cells (MDSCs). The MDSCs are heterogeneous population of cells, granulocytes (G-MDSC), and monocytics (M-MDSC), with potent immunosuppressant activity and may influence infectious diseases' outcomes [6, 7]. Conditions such as transplants, cancer, and some acute and chronic diseases were involved in MDSC enrichment and activation [7–10]. More recently, MDSC is becoming as essential cells in counter-balancing inflammatory responses and pathogenesis during infection [11].

MDSC release high levels of immunosuppressive cytokines such as interleukin (IL)-10, interferon (IFN)- γ and transforming growth factor (TGF)- β [8, 12–14]. Also, they inhibit T lymphocyte activity through various mechanisms. For example, M-MDSCs produce nitric oxide (NO) via inducible NO synthase (iNOS), whereas G-MDSCs produce reactive oxygen species (ROS), express arginine (Arg)-1 and reduce the levels of L-arginine [14–16].

In this context, annexin-A1 (ANXA1), a leukocyte regulatory and anti-inflammatory protein have been studied [17, 18]. Some works have described that ANXA1 expression in Treg cells could enhance its inhibitory function [19, 20]. Other work with transgenic mice deficient in ANXA1 indicates that T cell increased its effects on intracellular signalling, proliferation, and Th1/Th17 cytokine release [21]. However, no previous work has been described the presence of ANXA1 in MDSC.

Thus, this work aimed to identify the presence of MDSCs and the protein ANXA1 in leprosy patients with clinical forms of leprosy and with T1R and T2R.

2. Methods

2.1. Patients

Eligible leprosy patients were diagnosed with tuberculoid (TT), borderline (BB), lepromatous (LL) and with T1R and T2R (n = 170) in the years 2017 to 2019 in the Clinic of Infectious Diseases at the University Hospital Júlio Müller (UHJM) in Cuiabá, MT, Brazil. The patients were diagnosed according to the criteria established by Ridley and Jopling [3].

At the time of collection, the TT, BB and, LL patients were naïve of treatment. The T1R patients were under corticosteroid treatment. The T2R patients were under thalidomide treatment.

All T1R were clinically diagnosed as borderline-borderline. In T2R, 5 patients were borderline lepromatous, and 20 were lepromatous leprosy patients.

There were not included in this study individuals younger than 18 years and older than 70 years, pregnant or lactating women, seropositivity for HIV and co-infection with other parasitic diseases.

For data collection, a standard questionnaire was used with the following information: age, skin color, sex, characteristics of the lesion, region of the affected nerves, and the presence or absence of a Bacillus Calmette–Guérin (BCG) vaccine scar [22].

All patients were submitted to general physical and dermato-neurological examination by the physician responsible for the service. General health conditions, characterization of the lesion (location, size, edges, and thermal, painful and tactile sensitivity), evaluation of nerve thickening, and sensitivity tests in members through the Semmes-Weinstein monofilaments were evaluated [23]. Participants who agreed to participate in the study signed the informed consent form, approved by the Committee for Ethics in

Research of UHJM (CAAE No. 45051415.5.0000.5541), taking into account the Resolutions no. 466/12 of the Brazilian Health Council and international ethical guidelines (Declaration of Helsinki).

2.2. Collection of biological material

Tissue samples were collected at the time of diagnosis of leprosy. The procedure was initiated by the asepsis and local anesthesia with 2% lidocaine without vasoconstrictor, performing a biopsy using a "punch" of 4 mm at the edge of the lesion with a sign of clinical activity. The tissue fragment was immersed in 4% buffered (phosphate buffer saline, PBS) paraformaldehyde and transported to the Laboratory of Histology of the Faculty of Medicine, Federal University of Mato Grosso (UFMT), Cuiabá, Brazil, for diagnosis.

2.3. Histological analysis

The samples were washed in the same buffer, dehydrated in solutions with increasing ethanol concentration, cleared in xylene, and embedded in paraffin. Paraffin sections were obtained in the microtome HIRAX M60 (Carl Zeiss, Germany), placed on histological slides, rehydrated, and stained with hematoxylin-eosin for histopathological analysis. Another section was stained with Fite-Faraco, for acid-alcohol-resistant bacilli (BAAR) analysis. Morphological and quantification of the bacilli were done under a microscope. The results were expressed on a logarithmic scale of Ridley and Jopling [3].

2.4. Quantification of endogenous ANXA1 expression, and identification of M-MDSC and G-MDSC by immunofluorescence technique.

The detection of ANXA1 and cell markers in the MDSC were performed in skin biopsies of patients by immunofluorescence technique, according to Silva and collaborators [24]. For ANXA1 detection, the antibody rabbit anti-ANXA1 [Invitrogen, USA; 1:200 in PBS/bovine serum albumin (BSA) at 1%] was used.

For identification of M-MDSC, it was used a monoclonal mouse IgG anti-CD14 (SANTA CRUZ biotechnology, 1:100 in 1% BSA), rat anti-MHCII (sc-59318; Santa Cruz Biotechnology Inc., Santa Cruz, CA 1:100), goat anti-CD11b (1:50 in 1% BSA).

For identification of G-MDSC, it was used monoclonal mouse IgM anti-CD15 (SANTA CRUZ biotechnology, 1:100 in 1% BSA), mouse anti-MHCII (sc-59318; Santa Cruz Biotechnology Inc., Santa Cruz, CA 1:100), goat anti-CD11b (Abcam 1:50 in 1% BSA).

As secondary antibodies, it was used the following: goat anti-rabbit IgG conjugated to Alexafluor 488 fluorochrome (Invitrogen, USA, 1:200 in 1% BSA), goat anti-mouse IgG conjugated to Alexafluor 555 (Invitrogen, USA, 1:200 in 1% BSA), goat anti-mouse IgM conjugated to Alexafluor 555 (Invitrogen, USA, 1:125 in 1% BSA), goat anti-mouse IgG conjugated to Alexafluor 633 (Invitrogen, USA, 1:50 in 1% BSA), and donkey anti-goat IgG conjugated to Alexafluor 350 (Invitrogen, USA, 1:25 in 1% BSA). The secondary antibodies were incubated for 1 h at room temperature and in the darkroom.

Twenty fields were analysed in each patient dermis for MDSC analysis. Up to three cells per field were considered a low number. More than four cells per field were considered a high number. After identification, two blinded observers quantified the cytoplasmic content of ANXA1 using the Axiovision software (Carl Zeiss, GR) by optical density average. The expression quantification was measured according to the light spectrum, ranging arbitrarily from 0 to 255 (arbitrary units - a.u.). ANXA1 values were expressed as mean \pm standard error of the mean (SEM) in each MDSC.

2.5. Statistical analysis

The sample size was calculated using Software IBM SPSS Statistics version 22, considering a 90% confidence interval and sample power $> 80\%$. Population size was referred from data obtained at State Health Secretary of Mato Grosso. Statistical analyzes were performed using the chi-squared test and Fischer's exact test with a significance level of 5% ($p < 0.05$) for the estimated statistical associations. The ANXA1 results obtained were statistically compared with the aid of the software GraphPad Prism 5 (La Jolla, CA, USA) through the analysis of variance (Oneway ANOVA) with Bonferroni post-test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Clinical and histopathological data

The patients were evaluated by clinical data and bacilloscopy, being classified as follows: 40 patients TT, 40 BB, 40 LL, 25 T1R and, 25 T2R. Men were the predominant sex (55.9%), and brown was the predominant skin color (54.1%). The majority of patients (56.5%) were 40–59 years old (Table 1).

Table 1
Sociodemographic profile of leprosy patients.

Variables analyzed		Quantity	Percentage
Sex	Male	95	55.9%
	Female	75	44.1%
Skin color	Black	41	24.1%
	Brown	92	54.1%
	Caucasian	37	21.8%
Age	18–39	43	25.3%
	40–59	96	56.5%
	60–70	31	18.2%

3.2. Analyzes of the bacilloscopy index and BCG vaccination scar.

TT patients presented bacilloscopy index varied from 0 to 2+, and 62.5% have BCG vaccination scar. BB patients showed IB = 3 + to 4+, and 42.5% have BCG scar. LL patients showed IB = 5 + to 6+, and 40.0% have BCG scar. The patients T1R showed IB = 3 + to 4+, and 36.0% had BCG scar. The T2R had between BI = 4 + to 6+, and 48.0% had a BCG scar (Table 2).

Table 2
Analysis of the bacilloscopy index found in the tissue of patients with LL, T1R, and T2R with BCG scar.

		TT	BB	LL	T1R	T2R
0	Yes	8	-	-	-	-
	No	3	-	-	-	-
1+	Yes	8	-	-	-	-
	No	6	-	-	-	-
2+	Yes	9	-	-	-	-
	No	6	-	-	-	-
3+	Yes	-	15	-	6	-
	No	-	21	-	10	-
4+	Yes	-	2	-	3	8
	No	-	2	-	6	10
5+	Yes	-	-	12	-	3
	No	-	-	14	-	2
6+	Yes	-	-	9	-	1
	No	-	-	5	-	1
Total		40	40	40	25	25

3.3. M-MDSC and G-MDSC in LL, T1R, and T2R lesion with BCG vaccination scar.

The presence of M-MDSC (CD14⁺CD11b⁺MHCII^{+/-}) and G-MDSC (CD15⁺CD11b⁺MHCII^{+/-}) in skin lesions of patients with leprosy was evaluated. The majority of LL and T2R patients have a high number of M-MDSC and G-MDSC cells per field (66.6% and 50.0% in LL; 61.9% and 80.9% in T2R). T1R patients have a high number of M-MDSC (66.6%) in the skin lesion. The number of G-MDSC was not associated with the presence or absence of a BCG scar (Table 3).

Table 3
Quantity of MDSC in LL, T1R, and T2R with BCG vaccination scar.

		Cell number	BGC		<i>p</i>	
			Yes	No		
M-MDSC	BB	Low	3	3	-	
		High	0	0		
	LL	Low	6	6	0.338	
		High	16	12		
	T1R	Low	4	5	0.254	
		High	5	11		
	T2R	Low	2	6	0.031*	
		High	11	6		
G-MDSC	LL	Low	7	8	0.205	
		High	15	10		
	T1R	Low	7	12	0.438	
		High	2	4		
	T2R	Low	3	2	0.344	
		High	10	10		
	* <i>p</i> < 0.05					

3.4. Analyzes of ANXA1 expression in M-MDSC and G-MDSC

The presence of ANXA1 was observed in all MDSC (Fig. 1). M-MDSC in the LL has higher levels of ANXA1 (125.5 ± 4.1 u.a) when compared to the reactive patients (Table 4). Also, the ANXA1 expression in T1R was the lowest when compared to T2R (Table 4).

Table 4
 Analysis of ANXA1 expression in M-MDSC and G-MDSC at skin lesions of patients with leprosy.

	M-MDSC	G-MDSC
LL	125.5 ± 4.1	110.8 ± 5.7
T1R	54.2 ± 4.3***	109.0 ± 4.6
T2R	91.8 ± 4.3*** ###	106.1 ± 3.9
M-MDSC = *** $p < 0.0001$ compared with LL		
### $p < 0.0001$ compared with T1R		

The analysis of ANXA1 in G-MDSC (Fig. 1) showed that LL, T1R, and T2R have similar levels (ex: LL: 110.8 ± 5.7 u.a) (Table 4).

4. Discussion

This study identified the presence of MDSCs in patients with leprosy and its expression of ANXA1. This data was also correlated with the presence of the BCG vaccination scar and the bacilloscopy index to establish a possible default association in leprosy reactions.

The epidemiological data showed that men were the most affected in this study. These data are consistent with the findings reported in the literature [25–27]. Several factors contribute to this scenario: lower health care dispensed, lifestyle factors, less concern with the self. Altogether, it may contribute to late diagnosis and, subsequently, disease dissemination. Also, the data showed that the predominant skin color as brown is observed in other studies [25, 28]. Most of the participants were residents in the urban area, which is also corroborated in other studies [29].

Then, the presence of the BCG vaccination scar was evaluated. The majority of them have the BCG vaccination scar. However, it is not completely clear how efficient this vaccine can protect leprosy patients [30, 31]. Those with BCG scar presented a higher bacilloscopy index with bacilli (*M. leprae*) intact. Some studies postulate that genetic polymorphisms might be responsible for the immunological incapacity of protection [32].

Subsequently, the presence of MDSCs were reported in patients with leprosy. A higher number of MDSCs were observed in LL and T2R patients when compared to T1R ones. The literature suggested that infectious diseases might inhibit the maturation of myeloid cells in the bone marrow, inducing migration to the inflammatory site, and differentiation as suppressor cells [33]. The presence of these cells in patients LL alone can reduce the efficiency of the immunological system to fight against *M. leprae*. This data suggest that the fundamental role of MDSCs in the regulation of inflammatory reaction. Some

studies say M-MDSC induces the proliferation of macrophages M2-like in hypoxic tumour areas [13] and contributes to the extracellular matrix remodelling [34]. Also, M-MDSC produces reactive oxygen species, which disrupts the T-cell function by modifying its TCR- ζ chain [16]. Regarding the G-MDSC, it mediates an immunosuppressive pattern through STAT6 signalling and expression of ARG-1 and TGF- β [8]. Also, G-MDSC induces the activation of Treg cells through IFN- γ and IL-10 [12].

The presence of M-MDSCs in high number was observed in the patients with LL and T2R patients with BCG vaccination scar. Some studies have described that the BCG vaccination or inoculation with *M. tuberculosis* antigens induce the MDSC migration [35, 36]. This data and other genetic polymorphisms might make those patients more susceptible to developing the worst symptoms of leprosy.

Finally, to analyze a possible mechanism of action of MDSCs in patients with leprosy, the ANXA1 expression was analyzed. Previous studies have already demonstrated the ANXA1 expression in leukocytes of leprosy patients [37, 38]. However, this is the first study that highlights the presence of this protein in MDSCs. The data showed that the ANXA1 levels found in M-MDSCs were higher in LL patients when compared to the T1R and T2R, while similar levels were observed in G-MDSC at all patients. The literature shows that the ANXA1 is an endogenous regulatory protein expressed at high concentrations in granulocytes, particularly neutrophils [39–42]. The lower levels of ANXA1 in M-MDSCs of T1R and T2R might be due to drug treatment. This result can be an important limitation of this work. In particular, literature shows that, after 24 h of glucocorticoids treatment, ANXA1 expression reduces in macrophages [40]. Therefore, the high levels of this protein, observed in G-MDSC, might be linked to the type of cell lineage. It is well known that the ANXA1 has a modulatory role in the innate and adaptive immune response. Studies with ANXA1 knockout animals show the acute and systemic inflammation exacerbation by pro-inflammatory TNF- α , IL-1 β , and IL-6 release [17, 39, 40]. Also, ANXA1 is involved in the induction of IL-10 production (de Coupade et al., 2001; Parente and Solito, 2004; Damazo et al., 2005). This cytokine is produced by MDSCs and is an essential molecule in the immune system regulation. This data suggest that the high ANXA1 expression in MDSCs at LL and T2R patients may be related to the regulation of infectious response, reducing the effectiveness of T cells action, and establishing an anergic response, leading patient susceptible to *M. leprae*.

Clinical evolution of leprosy is directly involved with the participation of pro-inflammatory mediators, which direct the immune response to a cellular or humoral profile. Some of these have their role well elucidated in the literature, However, there are gaps related to issues of resistance or susceptibility to individuals exposed to the bacillus. It was demonstrated the presence and importance of MDSC that can influence the host response against leprosy. Many issues and challenges are still open for the research of MDSCs and their role in leprosy.

5. Conclusion

A high number of M-MDSC and G-MDSC was present in the skin lesions of patients with LL and T2R, whereas, in T1R patients, a high number of M-MDSC was observed. These data indicated a different

mechanism of recruitment of those cells dependent on immunological outcomes.

A higher number of M-MDSC was observed in LL and T2R patients with a BCG vaccination scar. These data may indicate an adjustment of the BCG vaccine in the recruitment of these cells in different clinical conditions of patients with leprosy.

LL patients expressed more ANXA1 in M-MDSCs than the patients T1R and T2R, possibly indicating the involvement of this protein in the anergic immune status of LL patients.

Abbreviations

List of abbreviations:

a.u; arbitrary units

ANXA1: annexin-A1

Arg: arginine

BAAR: acid-alcohol-resistant bacilli

BB: borderline

BCG: Bacillus Calmette–Guérin

BSA: bovine serum albumin

ENL: erythema nodosum leprosum

G-MDSC: granulocyte myeloid-derived suppressor cell

IFN- γ : interferon gamma

IL: interleukin

iNOS: inducible NO synthase

LL: lepromatous

MDSC: myeloid-derived suppressor cell

M-MDSC: monocytic myeloid-derived suppressor cell

NO: nitric oxide

PBS: phosphate buffer saline

ROS: reactive oxygen species

SEM: standard error of the mean

T1R: type 1 reaction

T2R: type 2 reaction

TGF- β : transforming growth factor beta

TT: tuberculoid

UHJM: University Hospital Júlio Müller

Declarations

Ethics approval and consent to participate

Participants who agreed to participate in the study signed the informed consent form, approved by the Committee for Ethics in Research of UHJM (CAAE No. 45051415.5.0000.5541), taking into account the Resolutions no. 466/12 of the Brazilian Health Council and international ethical guidelines (Declaration of Helsinki).

Consent for publication

The manuscript does not contain any individual person's data in any form.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contribution

SFS: Acquisition of data, Analysis and interpretation of data and Drafting the article; LCSC: Acquisition of data, Analysis and interpretation of data and Drafting the article; EAFJ: Acquisition of data; JMS: Analysis of data; JCL: Acquisition of data, Analysis of data; ASD: Conception and design of the study, Acquisition of data, Analysis and interpretation of data, Drafting the article, Final approval of the version to be submitted.

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Figures

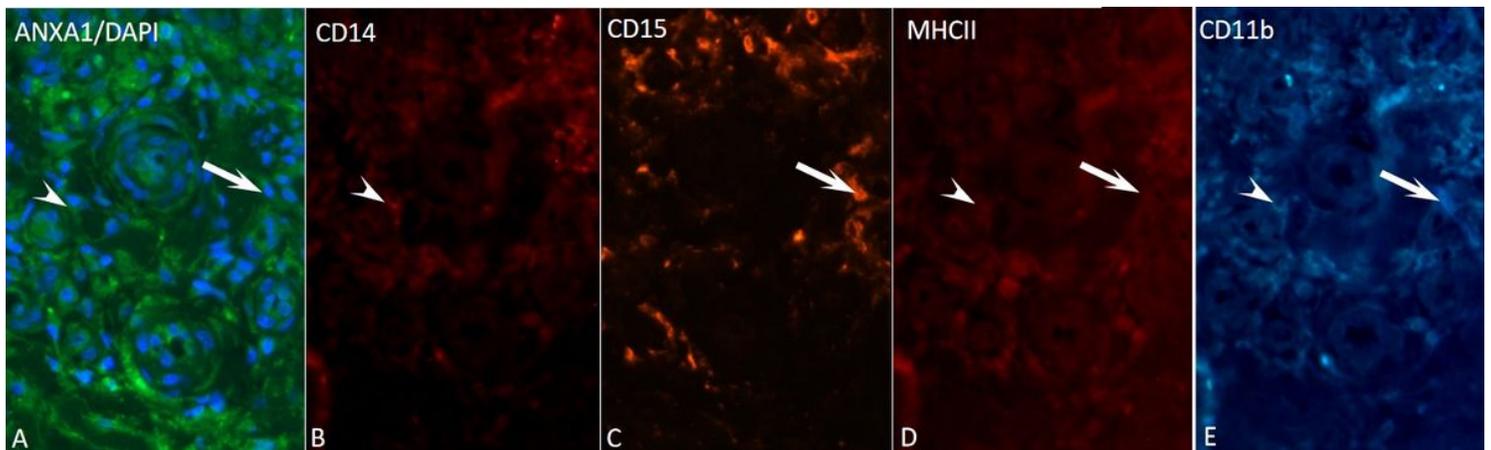


Figure 1

Analysis of immunofluorescence for ANXA1 in M-MDSC and G-MDSC in LL patient's skin lesion. ANXA1 (A), CD14+ (B), CD15+ (C), MHC-II+/- (D) and, CD11b+ (E) stain. M-MDSC (arrowhead) and G-MDSC (arrow). Bar = 20 μ m.

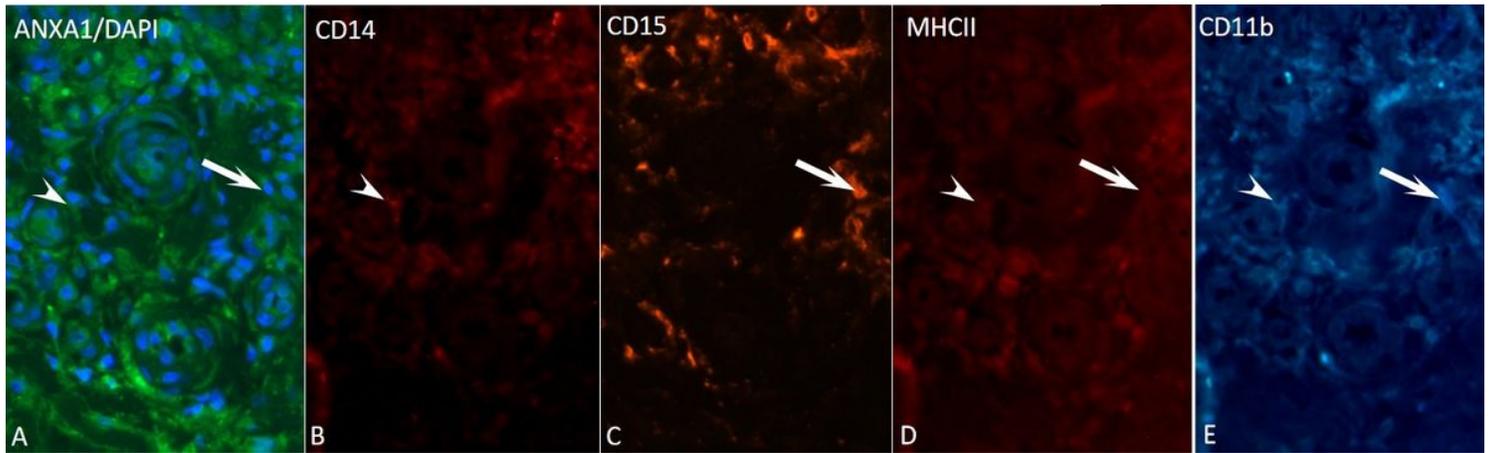


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