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HEALTH SCIENCES

Profile of interstitial cells of Cajal in a murine model of chagasic megacolon

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Abstract: Disorders of gastrointestinal motility are the major physiologic problem in chagasic megacolon. The contraction mechanism is complex and controlled by different cell types such as enteric neurons, smooth muscle, telocytes, and an important pacemaker of the intestine, the interstitial cells of Cajal (ICCs). The role of ICCs in the progression of acute and chronic Chagas disease remains unclear. In the present work, we investigate the aspects of ICCs in a long-term model of Chagas disease that mimics the pathological aspects of human megacolon. Different subsets of ICCs isolated from Auerbach's myenteric plexuses and muscle layers of control and *Trypanosoma cruzi* infected animals were determined by analysis of CD117, CD44, and CD34 expression by flow cytometer. Compared with the respective controls, the results showed a reduced frequency of mature ICCs in the acute phase and three months after infection. These results demonstrate for the first time the phenotypic distribution of ICCs associated with functional dysfunction in a murine model of chagasic megacolon. This murine model proved valuable for studying the profile of ICCs as an integrative system in the gut and as a platform for understanding the mechanism of chagasic megacolon development.

Key words: Chagas disease, interstitial cells of Cajal, megacolon, *Trypanosoma cruzi*.

INTRODUCTION

Trypanosoma cruzi was identified as the causative protozoan of Chagas disease (CD) more than 100 years ago. Yet, it remains a social and public health problem, mainly in endemic areas of 21 Latin American countries (World Health Organization 2010). The disease was a disease of Latin American rural populations for centuries. The exodus from rural towards urban areas has expanded the reach of illness. The non-vectorial transmission channels such as blood transfusion, congenital transmission, and organ transplants, have become critical to other countries and continents affecting international communities. In previous decades, Chagas disease was increasingly detected in the United States of America and Canada and many European and some Western Pacific countries due mainly to population mobility, mostly migration (World Health Organization 2019).

Chagas disease usually comprises an acute and a chronic phase. A proliferanting parasite in blood and tissue marks the acute phase. It presents asymptomatic, in most cases, or with mild symptoms, such as fever, fatigue, body aches, headache, rash, loss of appetite, diarrhea, and vomiting. Severe acute disease occurs in less than 5% of patients presenting acute myocarditis, pericardial effusion, and meningoencephalitis (Centers for Disease control and prevention 2022). Untreated patients remain chronically infected, most people never develop symptoms (indeterminate form) or show visceral

involvement characterized by cardiomyopathy or mega viscera (megaoesophagus, megacolon, or both) (Higuchi et al. 1993, Koberle & Nador 1955, Pérez-Molina & Molina 2017).

Megacolon is characterized by an increase in the thickness, dilation, and sometimes elongation of the intestinal wall. These changes include considerable damage to the enteric nervous system (ENS) and subsequent ganglionic and intramuscular denervation (da Silveira et al. 2007a, Koberle 1956, Ricci et al. 2020). The thickening of its wall accompanies the permanent dilation of a (colonic) gut segment. Initially, thickening predominates over dilation, whereas later, as a sign of decompensation, the gut wall distends with the muscle layers becoming thinner (Koberle 1968, Côbo et al. 2012). Muscle layer thickening is not only due to increased muscle tissue but also to the proliferation of connective tissue. Indeed, massive fibrosis has also been found in chagasic megacolon (Ricci et al. 2020, Campos et al. 2016, da Silveira et al. 2007b, Iantorno et al. 2007, Jabari et al. 2014).

The main symptom associated with megacolon is chronic constipation (Lopes et al. 1988, Bern et al. 2007, Meneghelli 1985, De Oliveira et al. 1998). Through manometry studies, it was shown that colonic basal motility is lower in chagasic patients compared to normal subjects. Some studies showed a reduced relaxation of the internal sphincter of the anus in chagasic patients (Adad et al. 2001, Cavenaghi et al. 2008, Meneghelli et al. 1982, Meneghelli 1985, Salvador et al. 2015). However, the mechanisms involved in CD-related intestinal dysmotility are poorly understood. In this scenario, peristaltic motor activity studies are essential to understanding the contractility activity coordination in the intestine (Huizinga & Lammers 2009, Dickson et al. 2007).

Classified as "pacemakers" of the gut, the interstitial cells of Cajal (ICCs) play critical roles in gastrointestinal motility and have been associated with a pathogenic role in CD. The ICCs are located in the lamina propria, submucosal and myenteric plexus, and the different muscle layers associated with the ENS (Figure 1). Several studies show the reduction of the ICCs population in megacolon of chagasic patients using immunohistochemistry and immunofluorescence technique (Adad et al. 2013, Alonso Araujo et al. 2012, Jabari et al. 2014). Interestingly, this such as Hirschsprung's disease, inflammatory bowel disease, and slow transit constipation (He et al. 2000, Wang et al. 2009, Farrugia 2008) ICCs also have distinct profiles based on the expression of specific markers, such as mature ICCs ($CD117^{+}CD44^{+}CD34^{-}$) and progenitors ICCs ($CD117^{low}CD44^{+}CD34^{+}$) (Lorincz et al. 2008). The phenotype profiles of progenitors ICCs are observed in the intestine in some experimental models (Lorincz et al. 2008, Li et al. 2019), and in human pathologies such as Hirschsprung's disease (Chen et al. 2014). Also, progenitor ICCs descriptions have been found in human CD studies and, other gastrointestinal motor disorders (Geraldino et al. 2006, de Lima et al. 2008, Jabari et al. 2013).

Injury in the ICCs associated with enteric nervous damage has been considered the cause of uncoordinated contractility activity in megacolon CD (Negreanu et al. 2008, Hasler 2003). Also, altered distribution of ICCs has been demonstrated in human chagasic megacolon (Adad et al. 2012, Geraldino et al. 2006, Hagger et al. 2000). However, these human studies were based on small, limited samples of megacolon and performed with only descriptive assays using immunohistochemistry technique. Here, we have focused on studying the profile of ICCs in the chronic phase using the flow cytometry platform. This powerful tool allows us to separate and characterize isolated ICC profiles in *T. cruzi* infection since the visualization of

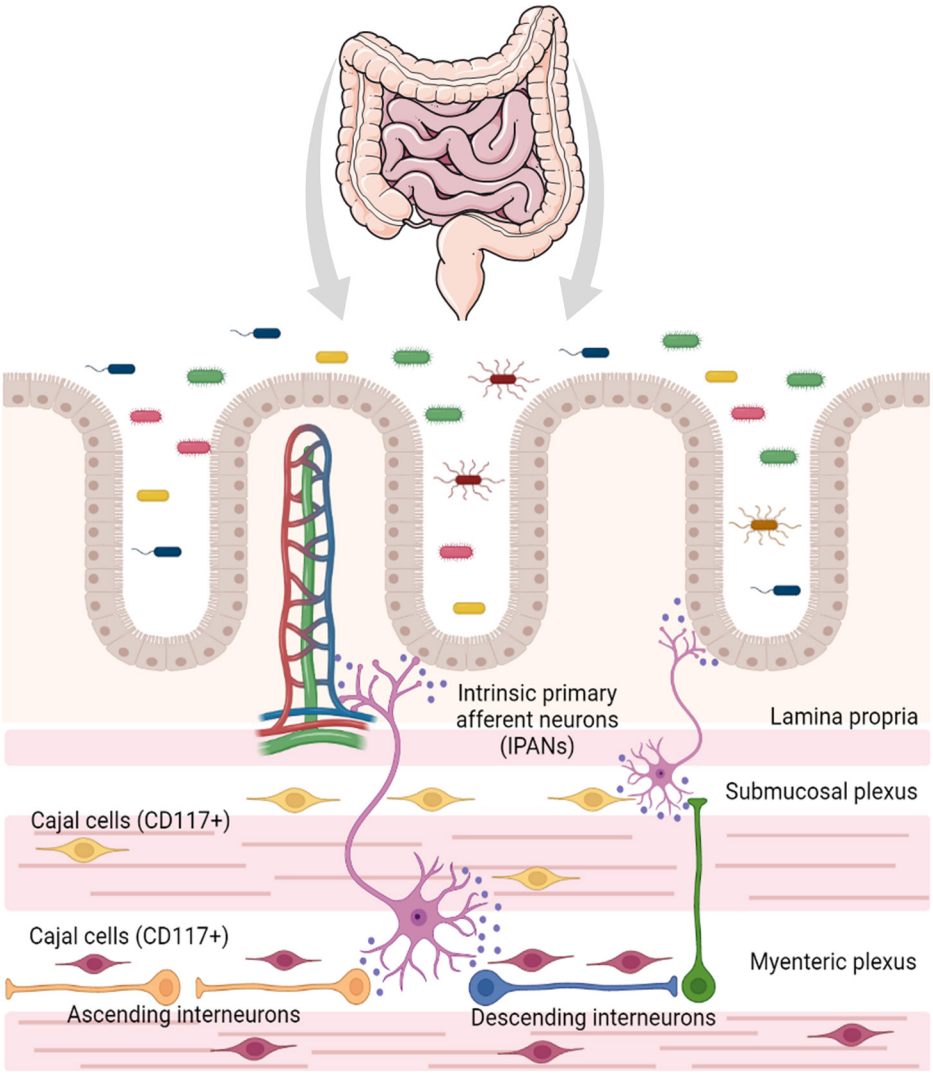

Figure 1. Ilustration of the distribution of ICCs in the *lamina propria*, submucosa and myenteric plexus in different muscle layers associated with the enteric nervous system (ENS). Created by https://www.biorender.com/.

these cells is not easily done in the histological sections. Finally, this work may partly explain the initial functional changes in the murine model of chagasic megacolon previously published by our group (Campos et al. 2016). In non-infected (control animals), we do not expect any changes in ICCs as documented in mice and human intestines by Klüppel et al. (1998) Thus, accurately identifying and quantifying the ICC profile in CD remains essential. We performed the first flow cytometry characterization to describe the ICC profile in a murine model of acute and chronic megacolon chagasic disease. A better description of these cells provides mechanistic insights into effective interventions to prevent morbidity associated with late manifestations of megacolon in CD.

MATERIALS AND METHODS

Ethics

In vivo, animal experiments complied with the principles established by guidelines and the Brazilian Practice Directive for the Care and Use of Animals for Scientific and Didactic Purposes (Notice MCTI No. 1 - CONCEA / MCTI). Animal studies were approved under protocol numbers 262/2016 and 31/2017.

Mice

Female Swiss mice, 4-week old, supplied by the Bioterium of the Institute of Biological Sciences of UFMG, kept in plastic cages in a room with controlled dry temperature (24°C) under a light / dark cycle of 14/10 h and access to water and conventional mouse food (Nuvilab® Nuvital, Brazil).

T. cruzi infection experimental protocols

Mice infection was performed by intraperitoneal (IP) injection of 50,000 blood trypomastigotes of *T. cruzi* Y strain, as described before (Brener 1962). The efficacy of the infection was evaluated by the presence of parasites in fresh blood four days after inoculation.

The animals were randomly divided into two groups, infected and non-infected controls. The non-infected control group was composed of a control acute phase (CAP) and a control chronic phase (CCP) group. The infected group was composed of the infected acute phase (IAP) group euthanized at 11 days post-infection (d.p.i) before manifesting disease signals. The infected chronic phase group (ICP) was treated orally with a single dose of benznidazole (Lafepe, Brazil), 500 mg/kg, at 11 d.p.i. and maintained up to 7 months post-infection (m.p.i.). The animals of ICP group were euthanized at 3 and 7 m.p.i. (ICP3 and ICP7 groups, respectively). The non-infected age-matched chronic control (CAP and CCP) groups were maintained in the same conditions and euthanized at appropriate months indicated as the control chronic phase (CCP3 and CCP7 groups, respectively).

Immunohistochemistry

Colon tissue slides from mice in the chronic phase (7 months) were immunostained. Briefly, the slides were incubated with primary anti-CD117 antibody (human, 1:500) (Abcam, USA) overnight at 4°C (Abdo et al. 2021, Pawlicki et al. 2019). Then, the primary antibody were detected using an anti-mouse/anti-rabbit detection system (Novolink Polymer Detection System; Leica Biosystems, Newcastle Upon Tyne, UK) according to the manufacturer's instructions. The sections were counterstained with diluted Harris Hematoxylin solution and permanently mounted with Entellan (Merck, USA).

Immunofluorescence and Photographic Documentation

Primary cultures of mouse enteric neurons (Ricci et al. 2020) submitted to the above-described conditions and grown on coverslips were fixed with 4% buffered paraformaldehyde. The slides were incubated with primary anti-CD117 antibody (human, 1:500) (Abcam, USA) overnight at 4°C. Identification of cell nuclei *in vitro* was performed by the fluorescence-emitting probe Hoechst H33342 (Invitrogen, USA), which binds to nuclear DNA and allows the optimal visualization of parasite and host nuclear morphology. The coverslips were analyzed under the Olympus BX51 fluorescence microscope and images were obtained using Image-Pro Express 4.0 software (Media Cybernetics, USA).

Isolation of cells in the inter-muscular layers (ICC-MY) in the murine model of chagasic megacolon

Isolate cells were performed as routinely done (Ricci et al. 2020). Briefly, after removal, the large intestine segment was placed in Krebs solution and sectioned along the mesenteric border and delaminated of the layers (mucosa, submucosa, and serosa) to expose the muscular layer where the Auerbach's myenteric plexuses were laid (Smith et al. 2013). The tissue was incubated for 15 minutes at 37 °C in 1mg/ml collagenase type II-S solution (Merck, USA). The fragments were centrifuged at 200 x *g* for 5 minutes at 4 °C and washed with Hanks/HEPES buffer solution for 5 minutes under stirring, followed

by centrifugation at 200 x *g* for 5 minutes at 4 °C. Trypsin solution (0.25%; Merck, USA) was added at 37 °C, followed by stirring for 15 minutes and manual homogenization. Next, the supernatant was removed and added to 1ml of sterile culture medium (Minimum Essential Medium; 31095-029; Gibco, Invitrogen, USA). The suspended cells were submitted to immunostaining of the cell markers and submitted to a subsequent acquisition of data in a flow cytometer.

Flow cytometry analysis

The cells were centrifuged at 3000 G for 5 min and resuspended in PBS (0.01 M phosphate-buffered saline, pH 7.2) with 0.5% Bovine Serum Albumin (BSA, Inlab, Brazil) and stained with anti-CD117 (BD Bioscience, USA), anti-CD45, anti-CD11b, anti-CD11c, anti-CD34, and anti-CD44 (eBiosciences, USA) (Table I), diluted in PBS with 0.5% BSA mix, fixed by a 45-min incubation at 4°C. The cells were washed two times in PBS-1% bovine serum albumin, fixed with a 4% paraformaldehyde solution (20 min), and examined in a flow cytometer. The data acquisition was performed by FORTESSA LSR cytometer using the DIVA software (BD Biosciences) at the Multi-User Flow Cytometry Platform (Instituto René Rachou/FIOCRUZ/MG). Analysis was performed using the Flow Jo, LLC program. The strategy used to identify the ICCs was using an exclusion gate (FITC) for anti-CD45, anti-CD11b, and anti-CD11c to exclude inflammatory cells and a positive gate for CD34, CD44, and CD117.

Statistical analysis

The statistical analyses were performed using the software GraphPad Prism (v 8.0) (GraphPad Software Inc., La Jolla, CA). The Shapiro-Wilk test revealed that the parameters evaluated did not show a significant departure from normal distribution. Comparisons between means were made using unpaired student's tests (i.e., Control *vs*. Infected). When a significant F-value was found, we performed a post-hoc test according to the coefficient of variation (CV): Tukey (CV ≤ 15%) or Student-Newman-Keuls (CV > 15%). The α level was set at 0.05. Data are shown as mean ± standard deviation (SD).

RESULTS

In vivo and *in vitro* evidence for the presence of ICCs

ICCs were observed in the immunohistochemically stained sections from the intestinal wall collected at the chronic phase at 7 months (Figure 2a, b). The ICC-IM (intramuscular) is scattered throughout the muscle and parallel smooth muscle cells in both muscle layers (black arrows). We also observed ICCs in a primary culture of enteric neurons using the immunofluorescence technique (Figure 2c, d; white arrows).

Table I. Fluorescent-labeled antibodies used for flow cytometry experiments.

Antibody	Clone	Vendor
BV605-conjugated anti-CD117	2B8	BD Biosciences
PE-conjugated anti-CD44	IM7	eBioscience
eFluor 660-conjugated anti-CD34	RAM34	eBioscience
FITC-conjugated anti-CD45	HI30	eBioscience
FITC-conjugated anti-CD11b	M1/70	eBioscience
FITC-conjugated anti-CD11c	N418	eBioscience

The description of the profile of Cajal cells in a chronic murine model of chagasic megacolon

Figure 3a shows the selection of ICCs population to be analyzed and the frequency of ICCs obtained by flow cytometry. ICCs were sub-gated into: $CD44^{+}CD117^{+}$ (undifferentiated cells), and the subtypes $CD44^{+}CD117^{+}CD34^{-}$ (mature ICCs) and $CD34^{+}CD117^{low}CD44^{+}$ (progenitor ICCs). There was a decrease in $CD44^{+}CD117^{+}$ cells in 3 and 7 m.p.i. compared to their respectively controls (p = 0.02) and (p = 0.0286) (Figure 3b). The ICCs were decreased in IAP and ICP3 compared to their respectively controls (p = 0.0286) and (p = 0.006) (Figure 3c). Regarding the ICCs progenitors ($CD117^{low}CD34^{+}CD44^{+}$), there are no differences between the groups (Figure 3d).

DISCUSSION

Loss of ICCs were associated with motor disorders of the gut. However, these cells' physiological and pathophysiological roles have not been clearly defined. Some authors described the reduction of ICCs in colonic biopsies in chagasic patients with megacolon. However, these studies do not discriminate the profile of ICCs (Hagger et al. 2000, Geraldino et al. 2006, Iantorno et al. 2007). For the first time, our study characterizes the subtypes of ICCs in the long-term murine model of chagasic megacolon. This work showed that infection with *T. cruzi* induces a transient loss of mature ICCs (11 d.p.i. and 3 m.p.i). These cells' decrease is associated with parasite induced acute inflammation (Campos et al. 2016, Ricci et al. 2020). The Y strain used in our experiments is partially resistant to benznidazole (BZ) (Khare et al. 2015). It acts directly against circulating trypomastigotes and intracellular amastigotes, but its effectiveness depends on the length of treatment, dosing, and disease phase (Filardi & Brener 1987, Marin-Neto et al. 2008, Pedrosa et al. 2001). So, the animals we treated with only a single dose of BZ at 500mg/kg of body weight improved their survival chances but developed a chronic infection. This protocol ensured the control of parasitemia and increased survival so that the animals that presented parasite-induced colonic wall inflammatory and degenerative

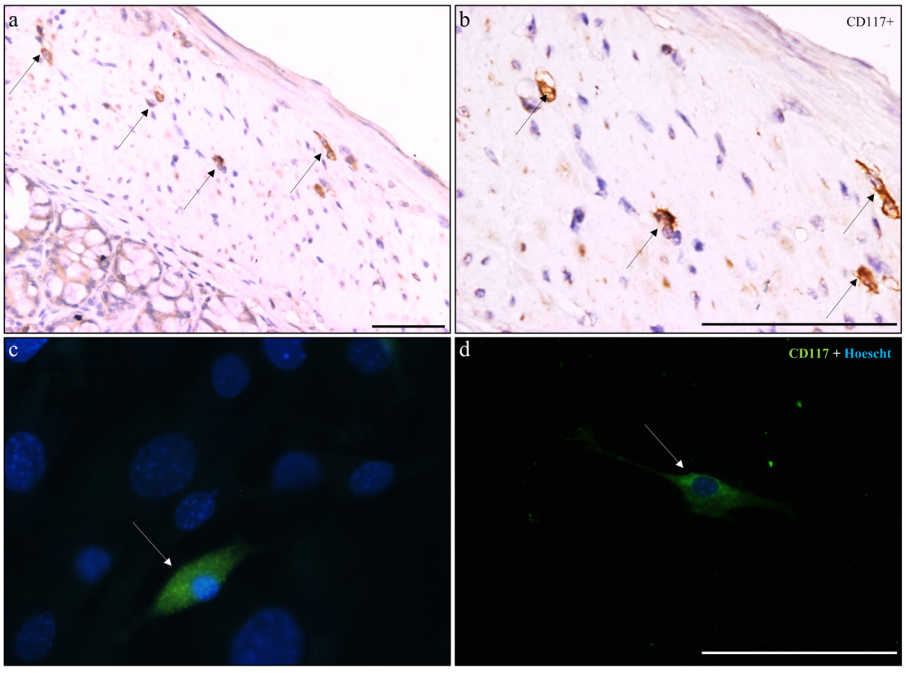

Figure 2. Distribution of ICCs in chronic colon tissue (7 months) and in a primary culture of myenteric neurons. a-b: Immunolabelling of paraffin-embedded tissues from colons at 7 months using anti-$CD117^{+}$ in the muscle and myenteric region (arrows). c-d: Immunofluorescence of primary cultures of mouse myenteric neurons using anti-$CD117^{+}$ (green) and nuclear staining with Hoechst 33342 (blue). Scale bar: 10µm. 20X and 40X objective. Data are representative of two independent experiments.

changes reached the chronic phase. The treatment of mice in the chronic phase with BZ was, therefore, not the aim of our study since we have a model (Campos et al. 2016, Ricci et al. 2020) that mimics the chagasic megacolon without any influence of BZ in this phase. Also, the development of megacolon requires severe denervation, preceding the establishment of dilation, and favoring the hypothesis that the reduction in ICC number is, in part, a consequence

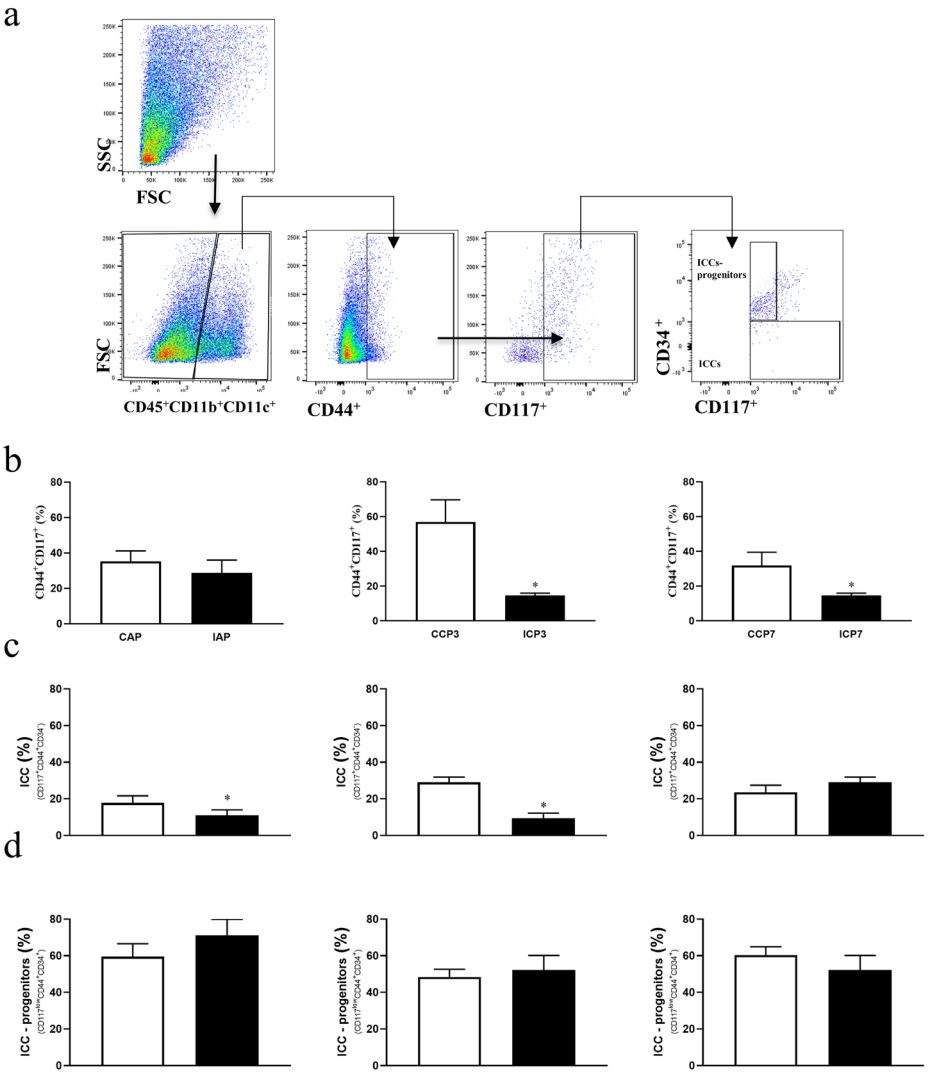

Figure 3. **Alterations of the frequency of ICCs in the murine model of chagasic megacolon. a: Representative dot plots from ganglia and intestinal muscle layer of Swiss mice infected with 50,000 *T. cruzi* strain Y trypomastigotes obtained from animals at the acute (CAP, controls; IAP, infected) and chronic (CCP, controls; ICP3, ICP7, months post-infection, respectively) phases, showing the gating strategy used to identify ICCs subsets. According to CD45, CD11b and c, CD117, CD34 and CD44 expression levels, the cells were sub-gated into: $CD44^{+}CD117^{+}$ (total ICCs), and their subtypes, $CD44^{+}CD117^{+}CD34^{-}$ (mature ICCs) and $CD34^{+}CD117^{low}CD44^{+}$ (progenitor ICCs). b, c and d: Frequency of ICCs subsets in total ICCs cells from mice infected, or not with *T.cruzi* analyzed by Flow cytometry using Flow Jo, LLC. Statistical analysis: Student *t*-test. Difference in relation to the control group in infected acute phase (p = 0.0286) and infected chronic phase 3 months (p = 0.006) (*) in the mature ICCs. Difference in relation to the control group, infected chronic phase 3 months (p = 0.02) and infected chronic phase 7 months (p = 0.0286) (*) in the ICCs progenitors. Data are representative of two independent experiments, except for the 7 group that were performed one time (n = 4). Data are shown as mean ± standard deviation (SD).**

of similar mechanisms of denervation (Adad et al. 2012) and potential plasticity of smooth muscle and Cajal cells, showing the importance of characterizing these subtypes of cells in the context of structural alterations for chagasic megacolon development.

Interestingly, in the later time point, the mature ICCs returned to the same frequency as the control group in the chronic phase of infection (7 m.p.i.), suggesting that mature ICCs have a considerable regenerative capacity in the long-term murine model of chagasic megacolon. These finds are also observed in other pathological conditions, which could restore their networks after partial mechanical obstruction (Chang et al. 2001), inflammation (Wang et al. 2005), and pyloric hypertrophy (Vanderwinden & Rumessen 1999). Also, it has been suggested that gastrointestinal stromal tumors (GIST) may originate from ICC precursors due to activating mutations of Kit (CD117) (Streutker et al. 2007). ICCs depend on stem cell factor (SCF) signaling via c-Kit to develop and maintain their functions (Klüppel et al. 1998, Wu et al. 2000, Rich et al. 2003). Thus, this activation could be a mechanism that matures ICCs in pathological conditions, including Chagas disease.

ICCs play a central role in the gastrointestinal system, generating slow electrical waves and contractile activity, mediating muscle interactions with the nervous system, establishing potential and tone, and developing a "pacemaker" role in various autonomic innervation organs (Farrugia 2008, Sanders et al. 2014). This work follows a previous study in which gastrointestinal rhythmicity was affected in a murine model of chagasic megacolon involving the lack of response of chronically infected animals related to changes in the cholinergic responses modified by the installation of the megacolon (Ricci et al. 2022). Altogether, these results show that it is essential to consider the multiple aspects of the complex CD development, including the alterations in the ICC profile that associate with functional disorders in a murine model of chagasic megacolon.

This model is a valuable tool for better characterization of the ENS impairment mechanisms of intestinal CD with an emphasis on ICCs profile role looking for an integrative comprehension of acute and chronic phases pathogenetic mechanisms of Chagas disease.

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