PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

NOD2 receptor is crucial for protecting against the digestive form of Chagas disease

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Abstract

Digestive and cardiodigestive forms of Chagas' disease are observed in 2% to 27% of the patients, depending on their geographic location, *Trypanosoma cruzi* strain and immunopathological responses. The aim of this work was to evaluate the role of NOD2 innate immune receptor in the pathogenesis of the digestive system in Chagas' disease. Patients with digestive form of the disease showed lower mRNA expression of NOD2, higher expression of RIP2 and α-defensin 6, compared to indeterminate form, detected by Real-time PCR in peripheral blood mononuclear cells. In addition, there was a negative correlation between the expression of NOD2 and the degree of dilation of the esophagus, sigmoid and rectum in those patients. The infection of NOD2$^{-/-}$ mice with *T. cruzi* strain isolated from the digestive patient induced a decrease in intestinal motility. Histopathological analysis of the colon and jejunum of NOD2$^{-/-}$ and wild type C57BL/6 animals revealed discrete inflammatory foci during the acute phase of infection. Interestingly, during the chronic phase of the infection there was inflammation and hypertrophy of the longitudinal and circular muscular layer more pronounced in the colon and jejunum from NOD2$^{-/-}$ animals, when compared to wild type C57BL/6 mice. Together, our results suggest that NOD2 plays a protective role against the development of digestive form of Chagas' disease.

Author summary

Chagas disease is caused by the protozoan *Trypanosoma cruzi*, during the chronic phase of infection 2–27% of patients develop digestive form of the disease (megaesophagus and megacolon) that contributes to patient morbidity and mortality, generating costs for public health services, and especially affecting significantly the life quality of the patients.

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Although is known that many factors inherent of the parasite (tropism, genetics, virulence and antigenicity), host (age, gender, nutritional status, genetics and immune response) and geographical distribution may influence the development of the different clinical forms of Chagas disease, the exact mechanism that leads to megacolon and megaesophagus development are unknown. Here we showed that patients with digestive form of Chagas' disease do not express the innate immune receptor NOD2. By isolating a parasite from a digestive patient and infecting NOD2-deficient mice we observed a reduced intestinal motility, chronic development of colon and jejunum wall thickness associated with increased inflammatory mediators in the organ, when compared to wild type animals. Our results indicate that the NOD2 receptor protects against the development of the digestive form of Chagas disease and could be used as a biomarker for the development of gastrointestinal changes during *T*. *cruzi* infection in patients.

Introduction

Chagas disease is caused by *Trypanosoma cruzi* and affects 6–7 million people mainly in Latin America [1]. Patients chronically infected can remain asymptomatic (39–78%), characterizing the indeterminate chronic form of the disease, or develop cardiac (17–50%), digestive (3–27%) or cardiodigestive (2–11%) forms. In the digestive form of the disease, there is mainly involvement of the esophagus and colon, due lesions in the intramural enteric nervous system, tissue parasitism and chronic inflammation leading to the appearance of megaesophagus and megacolon, respectively [2–13].

Pathological alterations of megacolon and megaesophagus include hypertrophy of the circular muscular layer, focal inflammatory reactions in the vicinity of the myenteric plexus and in the muscular layer, and fibrosis of the myenteric plexus [14]. Megaesophagus and megacolon are generated when the denervation of myenteric plexus exceeds a critical level of, at least, 85% and 50%, respectively [15–17]. It is suggested that smooth muscles are sensitive to different stimuli, and the absence of moderating action of the myenteric plexus would make the smooth muscle hyperactive, contracting disorderly what could lead also to wall hypertrophy altering the motility of several segments of the digestive tract, in particular the esophagus and colon [18–20]. In mice models proinflammatory cytokines such as IL-12, IFN-γ and TNF-α are responsible for induction of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by macrophages, which contribute to the neurotoxic effects of the disease [21–25]. Denervation occurs in an irregular way and in variable intensity, due to factors related to the parasite and to the host, which have not yet been completely elucidated [26, 27].

Our group demonstrated increased of TLR8 and IFN-β expression in peripheral blood mononuclear cells of patients with digestive and cardiodigestive form of the disease [28]. TLRs activation leads NF-κB activation and production of inflammatory mediators, such as pro-IL1β, pro-IL-18, IL-6, IL-12 and TNF-α [29]. This inflammatory process contributes for the mechanism of myenteric neuronal reduction leading to megacolon and megaesophagus formation [30]. Crohn's disease and ulcerative colitis present an increase in the expression of TLR8 and inflammatory cytokines, such as type I interferon, IL-1β, IL-6, IL-12 and TNF-α [31–34]. Moreover, several studies have demonstrated that the susceptibility to inflammatory bowel diseases is related to NOD2 deficiency [35–39], what led us to investigate the role of NOD2 in the development of morphological inflammatory-induced alterations of the digestive system in Chagas' disease.

The NOD2 molecule is an innate immune receptor, expressed in the cytoplasm of macrophages, dendritic cells and Paneth cells, where it maintains intestinal homeostasis by binding mainly muramyl dipeptide (MDP), present in all types of bacteria [37, 38], and inducing the production of antimicrobial peptides, such as α-defensins (5 and 6) through the RIP2 signaling. In fact, the absence of NOD2 in mice and patients leads to the presence of potentially invasive bacteria, generating more frequent lesions in digestive epithelial tract than those with normal NOD2 expression [40–43].

Although studies have demonstrated the importance of inflammation and the presence of the parasite in the esophagus and colon in the pathophysiological process of megacolon and megaesophagus generation in chronic chagasic patients [14, 44–46], the mechanism by which some patients develop the anatomopathological alterations is unclear. In this study, we have demonstrated the importance of the NOD2 receptor in protecting against the inflammatory component of digestive form of Chagas' disease. The better understanding of the pathophysiological mechanisms involved in the genesis of megaesophagus and megacolon can lead to reduction in the morbidity and mortality associated with the digestive form of Chagas' disease.

Methods

Ethics statement

All patients included in this study signed the written informed consent form. The study was performed according to human experimental guidelines of the Brazilian Ministry of Health (Resolution 466/12-CNS/MS) and the Helsinki Declaration. This study was approved by the Ethics and Research Committee of the State University of Rio Grande do Norte (UERN) protocol No. 027.2011, and a Certificate of National System of Ethics in Research (CAEE-SISNEP) with protocol number 0021.0.428.000–11.

All procedures and experimental protocols performed with animals were conducted in accordance with the directives issued by the Brazilian College of Animal Experimentation (COBEA). The animal experimental protocol was approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Rio Grande do Norte (UFRN), protocol No. 23/2015.

Patient study population

The population consisted of 80 individuals aged 18 to 79 years old. Population was selected from 10 municipalities in the Rio Grande do Norte state in Brazil: Alexandria, Apodi, Caraúbas, Governador Dix-Sept Rosado, Ipanguaçu, Mossoró, Pendências, Rodolfo Fernandes, Serra do Mel and Severiano Melo. All the individuals were submitted to serological screening for *T*. *cruzi* infection analysis. Indirect hemagglutination (Chagatest), recombinant ELISA (Wiener Lab, Rosário, Argentina) and in house indirect immunofluorescence reaction with epimastigotes of *T*. *cruzi* strain Y [47] were performed. Samples with inconclusive results were submitted to Western blot confirmatory serology (TESAcruzi, BioMérieux, Brazil) [48]. The results were considered positive when the sample was reactive in at least two of the selected methods, according to the recommendations of the World Health Organization and the Brazilian Consensus on Chagas disease [5].

Positive patients were clinically evaluated and the clinical forms were characterized using electrocardiogram (ECG), two-dimensional transthoracic echocardiography, chest X-rays and contrasted esophagus and colon (opaque enema). Esophageal contrast radiography was performed in the right anterior oblique position using barium sulfate (Bariogel, Cristália Laboratory, Brazil). Esophagus changes were classified in four levels: I) unchanged caliber, discreet contrast retention; II) small/moderate increase of the caliber, contrast retention and tertiary

waves; III) large caliber increase, hypotonia, poor motor activity and great retention of radiological contrast; IV) elongated esophagus lying over the diaphragm with great retention of radiological contrast—dolico-megaesophagus [49]. Contrasted colon radiographs were performed in the supine, ventral and right lateral position using barium sulfate solution via the rectum without prior bowel preparation or double contrast use [50]. The diameter of the rectum was measured in the right lateral decubitus position, and the sigmoid colon was measured in the dorsal decubitus position, or in the ventral decubitus position if necessary [7, 51].

Patients with electrocardiographic and/or echocardiographic alterations suggestive of chagasic cardiomyopathy were evaluated by Holter 24h. Thus, asymptomatic chagasic patients who presented electrocardiograms, chest X-rays, and normal esophageal and colon contrasts were categorized with undetermined clinical form (IND, n = 18). Patients who presented with electrocardiographic abnormalities suggestive of cardiac involvement, symptomatic or not, and or cardiomegaly at chest x-ray were classified as cardiac (CARD, n = 17). Patients with altered esophageal and/or colon imaging (DIG, n = 15) and individuals with cardiomyopathy associated with megacolon and/or megaesophagus with cardiodigestive form (CARDIG, n = 15). Fifteen healthy individuals with similar age were used as controls. Data characterizing the chagasic population are shown in Table 1.

Parasites and mice experimental infection

The RN25 isolate of *T*. *cruzi*, was obtained from hemoculture by prof. Antonia C.J. Câmara in 2013 from a 65-year-old patient with chronic digestive form, from Serra Negra do Norte city, Rio Grande do Norte state, Brazil [52]. This is the same area where we recruited the patients included in this study. Unfortunately, this patient died due complications during a surgery to remove part of the large intestine, in 2013, before this study started.

Specific Pathogen-Free NOD2 knockouts ($NOD2^{-/-}$) [53] and their wild type controls C57BL/6 (WT) females with six weeks old and 20–25g of bodyweight were obtained from the Center for Special Mice Breeding at Ribeirão Preto Medical School (FMRP-USP). Mice were maintained in the Experimentation Bioterium at Health Sciences Center of the Federal University of Rio Grande do Norte, housed in the same room under controlled temperature (25 ˚C) and with a 12 h light/dark cycle. The animals were maintained in separated cages but in the same ventilated racks with filtered air, sterile water and food provided *ad libitum*. These animals were subjected to the same environmental bacteria.

Animals were intra-peritoneally inoculated with 1×10^3 trypomastigotes of RN25 isolate (TcII) [52]. Mice were euthanized during the acute phase (19^{th} day after infection, the parasitemia peak) or in the chronic phase (12 months after infection).

Parasitemia, survival and gastrointestinal motility

Parasitemia was determined by the method described by Pizzi [54] modified by Brener [55]. Five microliters of blood were collected from the tip of the tail and the count was performed under the optical microscope (400 ×), using laminula (22 × 22mm) in 50 random fields. Mortality was evaluated daily. Animal intestinal motility was analyzed 0, 15, 30, 90, 180 and 360 days after infection. Briefly, three hours after food deprivation, 0.3 mL of 10% aqueous suspension of charcoal in water was administered orally by gavage. The animals were observed at 5 min intervals until faeces with charcoal were eliminated (maximum time of observation was 450 min) [56, 57]. Ten animals from each group were used for these experiments, results were calculated based on evacuation time and expressed as mean ± standard deviation (SD).

Table 1. Clinical characteristics of chronic chagasic subjects from Rio Grande do Norte State included in this investigation.

	Indeterminate	Cardiac	Digestive	Cardiodigestive	Total
Gender					
Female	8/18 (44.4%)	6/17 (35.2%)	10/15 (67.7%)	5/15 (33.3%)	29/65 (40%)
Male	10/18 (55.5%)	11/17 (64.8%)	55/15 (33.3%)	10/15 (67.7%)	36/65 (60%)
Age (years± SD)	41.4± 10.7	49.7± 11.8	57.6± 8.9	65.9 ± 10.6	53.3± 10.2
Chest Radiography					
Cardiomegaly	0/18 (0%)	5/17 (29.4%)	0/15 (0%)	5/15 (33.4%)	10/65 (15.4%)
Cardiothoracic index (heart lateral diameter/chest lateral diameter ± SD)	0.43 ± 0.05	0.48 ± 0.05	0.50 ± 0.05	0.42± 0.03	N.A.
Contrast Radiography					
Megacolon	0/18	0/17	8/15 9 (53.3%)	9/15 (60%)	17/65 (26.1%)
Megaesophagus	0/18	0/17	3/15 (20%)	3/15 (20%)	6/65 (9.3%)
Megacólon + Megaesophagus	0/18	0/17	3/15 (20%)	4/15 (26.6%)	7/65 (10.7%)
Sigmoid size (cm± SD)	4.41±0.57	4.5±0.71	7.72±4.6	7.96±2.87	N.A.
Rectum size (cm± SD)	4.89±0.81	5.72±0.89	7.5±31	6.36±1.89	N.A.
Echocardiogram					
Left ventricular ejection fraction (% ± SD)	64.6 ± 3.42	55.8 ± 14.96	56.2 ± 13.84	65.0 ± 6.48	N.A.
Left ventricular mass index (g/m^2 body surface ±SD)	97.6 ± 21.6	100.6 ± 19.8	126.5 ± 54.4	95.9 ±16.8	N.A.
Left ventricular diastolic diameter (mm ± SD)	49.4 ± 2.9	50.6 ± 6.8	51.0 ± 5.8	47.6 ± 3.6	N.A.
Left ventricular aneurysm	0/18 (0%)	3/17 (17.7%)	1/15 (6.7%)	4/15 (26.7%)	8/65 (12.3%)
Electrocardiogram					
Right Branch Block	0/18 (0%)	5/17 (29.5%)	4/15 (26.8%)	0/15 (0%)	N.A.
Left Branch Block	0/18 (0%)	1/17 (5.9%)	1/15 (6.7%)	0/15 (0%)	N.A.
Anterosuperior divisional block	0/18 (0%)	4/17 (23.6%)	3/15 (20.1%)	0/15 (0%)	N.A.
Atrioventricular Block	1/18 (5.6%)	3/17 (17.7%)	2/15 (13.4%)	1/15 (6.7%)	N.A.
Supraventricular extrasystoles	1/18 (5.6%)	1/17 (5.9%)	1/15 (6.7%)	0/15 (0%)	N.A.
Ventricular extrasystoles	0/18 (0%)	3/17 (17.7%)	4/15 (26.8%)	0/15 (0%)	N.A.
Ventricular repolarization change	1/18 (5.6%)	3/17 (17.7%)	1/15 (6.7%)	1/15 (6.7%)	N.A.
Low voltage of the QRS	0/18 (0%)	8/17 (47.2%)	1/15 (6.7%)	2/15 (13.4%)	N.A.

SD: standard deviation; N.A.: not applicable; % percentage

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Quantification of inflammatory mediators by Real time PCR

RNA was extracted from peripheral blood mononuclear cells (PBMC) from patients and control group. Mice had RNA extracted from the colon in the acute and chronic phases (19th day and 12 months after infection, respectively). The RNA was purified using Total RNA Isolation System kit (Promega, USA), Trizol Reagent (Invitrogen, USA) and DNAse treatment. Purified RNA was stored at -80˚C. RNA concentration and quality were analyzed using Nanodrop 2000 (Thermo Scientific, USA). cDNA was synthesized from 2μg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). The reaction cycles were 10 min at 25˚C, 120min at 37˚C, 5min at 85˚C and infinite at 4˚C. PCR reactions were performed using the SYBR Green system in a 7500 Fast Real time thermal cycler (Applied Biosystems, USA). Standard Real Time PCR conditions were as follows: 50˚C (2 min) and 95˚C (10 min) followed by 40 cycles of 94˚C (30 s), variable annealing primer temperature (Table 2) (30 s), and 72˚C (1 min). The human specific primers (NOD1, NOD2, RIP2, α-defensin 5 and α-defensin 6) and mice (TLR2, TLR4, IL-10, IL-17, T-bet, TNF-α, IFN-γ, iNOS, defensin-A) used are described in Table 2. The expression mRNA levels were determined using the mean Ct values from triplicate measurements to calculate the relative expression levels of the target

Table 2. Sequences of the primers used for RT-PCR reactions.

Targets	Sense and antisense sequences	Primer annealing temperature
Hu- β-actin[1]	TGACTCAGGATTTAAAAACTGGAA CACATTGTGAACTTTGGG	56.5˚C
NOD1[1]	GTGGACAACTTGCTGAAGAATGAC CTGTACCAGGTCCAGAATTTTGC	60.2˚C
NOD2[1]	GCCACGGTGAAAGCGAAT GGAAGCGAGACTGAGCAGACA	59.6
RIP2[1]	TGCCACCTGAAAACT-ATGAACCT ACACTTCCCATGTGATAACTGCAT	58.4
α-Defensin 5[1]	GCCATCCTTGCTGCCATT GCTTCTGGGTTGTAGCCTCATC	59.6
α-Defensin 6[1]	CCACTCCAAGCTGAGGAT CTCTGCAAAGGAGACGGC	58.4
GAPDH[2]	TGCAGTGGCAAAGTGGAGAT CGTGAGTGGAGTCATACTGGAA	58.8
TLR2[2]	CGAGTGGTGCAAGTACG GGTAGGTCTTGGTGTTCATTATC	57.7
TLR4[2]	CCTCTGCCTTCACTACAGAGACTTT GGATCATTTCCGATAAGGCT	60.9
IL-10[2]	TGGACAACATACTGCTAACC GGATCATTTCCGATAAGGCT	55.7
IL-17[2]	AGTTTGGGACCCCTTTACAC TCTCATCCAGCAAGAGATCC	57.8
T-bet[2]	CCCACAAGCCATTACAGGATG TATAAGCGGTTCCCTGGCATG	59.9
TNF-α[2]	TGTGCTCAGAGCTTTCAACAA CTTGATGGTGGTGCATGAGA	56.9
IFN-γ[2]	GCATCTTGGCTTTGCAGCT CCTTTTTCGCCTTGCTGTTG	57.6
iNOS[2]	CGAAACGCTTCACTTCCAA TGAGCCTATATTGCTGTGGCT	56.7
Defensin A[2]	GGTGATCAGCATACCCCAGCATCAGT AAGAGAAAACTACTGAGGAGCAGC	57.5

[1]Human primer,

[2] Mouse primer.

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genes in the chagasic patients or infected mice compared to those in the healthy subjects or uninfected mice, respectively, and were normalized to the housekeeping gene β-actin (human) and GAPDH (mouse) using the $2^{-\Delta\Delta Ct}$ formula. The qPCR experimental protocol was described in previous work [28].

Histopathological analysis

Ileum, jejunum and colon of the animals were sectioned longitudinally for content removal, placed in Bouin's solution 2% acetic acid for 10 minutes for pre-fixation as described by Arantes & Nogueira [58]. The pieces were rolled and fixed in 10% formol diluted in PBS for 24 hours. The rolls were routinely processed and paraffin embedded. Sections with 4 μm were obtained by microtomy and mounted in slides for Hematoxylin and Eosin (HE) staining. The slides were analyzed and images photographed by using the optical microscope (Olympus BX 51) equipped with Image-Pro Express 4.0 software (Media Cybernetics, MO, USA). Images were analyzed in the KS300 software (Zeiss, Jena, Germany) to determine the thickness of the

jejunum and the colon muscular layer (μm). We obtained the average of three measurements for each one of 15 images obtained with the 20× objective from each animal. Semi-quantitative analysis was performed to determine inflammation and parasitism in colon tissue of C57BL/6 and NOD2$^{-/-}$ in the acute (19 days after infection) and chronic (12 months after infection) phases. Total number of inflammatory foci (characterized by the presence of at least 10 inflammatory cells) and amastigote nests in the intestinal muscle layer were quantified in 30 fields (200× magnification) from each animal with an optical microscope (Olympus BX51). Six animals for each group were used for histopathology and the PCR experiments.

Statistical analysis

All analyzes were performed on PRISM 9.0 software (GraphPad, San Diego, CA, USA). The Agostino-Pearson and Kolmogorov-Smirnov tests were used to verify the distribution of the data. ANOVA and Tukey-Kramer tests were performed to verify the difference between the groups with normal distribution, in the samples with non-parametric distribution the Kruskall-Wallis and Dunns test were used. The correlations of parameters analyzed in the patients were determined by the Spearman test. Differences were considered significant when $p < 0.05$.

Results

Digestive form of Chagas disease is correlated to deficient NOD2 expression in patients

Chagasic patients (n = 65) were classified as indeterminate (n = 18), cardiac (n = 17), digestive (n = 15) and cardiodigestive (n = 15) clinical forms according to electrocardiogram (ECG), two-dimensional transthoracic echocardiography, chest X-rays and contrasted esophagus and colon (opaque enema) analysis (Table 1). Aiming to analyze the expression level of the intracellular receptors NOD1 e NOD2, its adapter molecule and products induced, a real time PCR was performed in the PBMC samples from all groups of patients. Chagasic patients with the indeterminate, cardiac, digestive and cardiodigestive clinical forms of the disease had similar NOD1 mRNA expression (Fig 1A). On the other hand, patients with the digestive and cardiodigestive forms presented absence or drastic reduced expression of NOD2 mRNA, when compared to those with indeterminate and cardiac forms (Fig 1B). Curiously, those same patients expressed higher levels of RIP2 mRNA compared to indeterminate and cardiac Chagas' disease patients (Fig 1C). Furthermore, mRNA expression of α-defensin 6 is higher in digestive than indeterminate patients, which showed similar levels than cardiac and cardiodigestive patients (Fig 1D). All group analyzed had similar expression of α-defensin 5 transcripts (Fig 1E).

Digestive and cardiodigestive patients had the esophageal dilation, sigmoid dimension and rectum size measured by contrasted esophagus and colon (opaque enema). A negative correlation was observed between the expression of NOD2 mRNA and the degree of esophageal dilation ($R = -0.7978$; $p = 0.0044$) (Fig 2A), the sigmoid colon dimension ($R = -0.6109$; $p = 0.0177$) (Fig 2B) and rectum size ($R = -0.6365$; $p = 0.0166$) (Fig 2C) in those patients. Together, the data indicate that the deficiency in NOD2 expression is found in chagasic patients with dilation of the esophagus, colon and rectum.

NOD2$^{-/-}$ mice has reduced intestinal motility and hypertrophy of the jejunum and colon during chronic phase of *T. cruzi* infection

To analyze the role of NOD2 receptor in the gastrointestinal lesion development during *T. cruzi* infection, we took advantage from the experimental model in transgenic mice. The RN25 *T. cruzi* isolated from a patient with a digestive form of Chagas Disease in a previously work

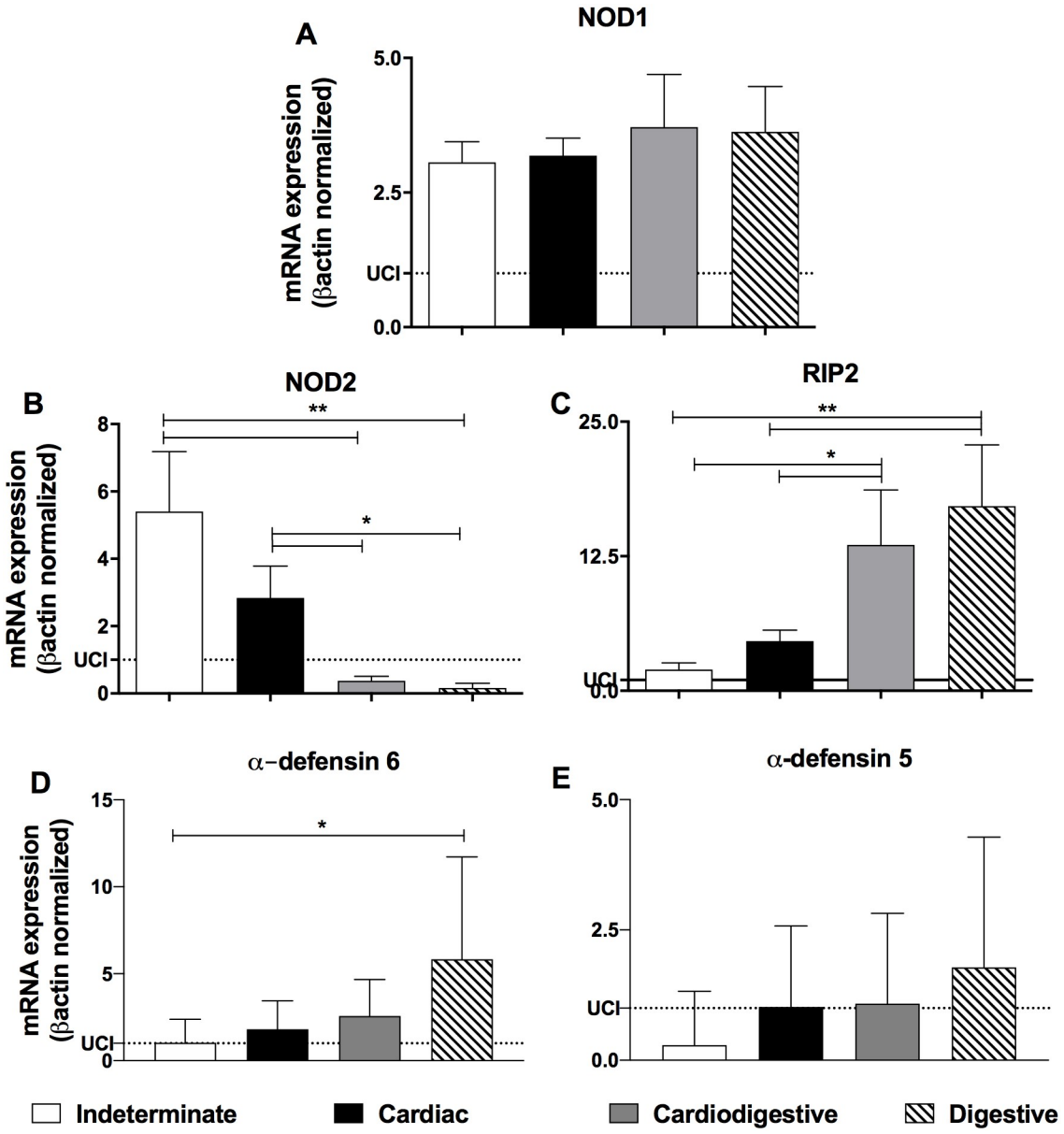

Fig 1. Patients with digestive form of Chagas disease showed low NOD2 and high RIP2 and α-defensin 6 expression. The mRNA expression levels of NOD1 (A), NOD2 (B), RIP2 (C), α-defensin 6 (D) and α-defensin 5 (E) were determined by real-time PCR in peripheral blood mononuclear cells of patients with the indeterminate (n = 18), cardiac (n = 17), cardiodigestive (n = 15) and digestive (n = 15) clinical forms of Chagas disease. The expression levels were normalized to the expression level of β-actin. The results are expressed as the means ± standard errors. $^{*}p < 0.05$; $^{**}p < 0.01$. Dotted lines represent uninfected control individuals (UCI, n = 15).

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[52] was inoculated in NOD2-deficient mice in an attempt to reproduce gastrointestinal changes observed in Chagas'patients with digestive clinical form. C57BL/6 mice were used as wild type (WT) control. Parasitemia, survival and gastrointestinal motility were evaluated. $NOD2^{-/-}$ mice presented higher parasitemia than C57BL/6 animals between the 21st and 30th day after infection (Fig 3A). Patent parasitemia was observed up to 40 and 50 days in the C57BL/6 and $NOD2^{-/-}$ infected mice, respectively (Fig 3A). However, 100% of survival was observed in both $NOD2^{-/-}$ and C57BL/6 mice infected by *T. cruzi*. $NOD2^{-/-}$ mice showed

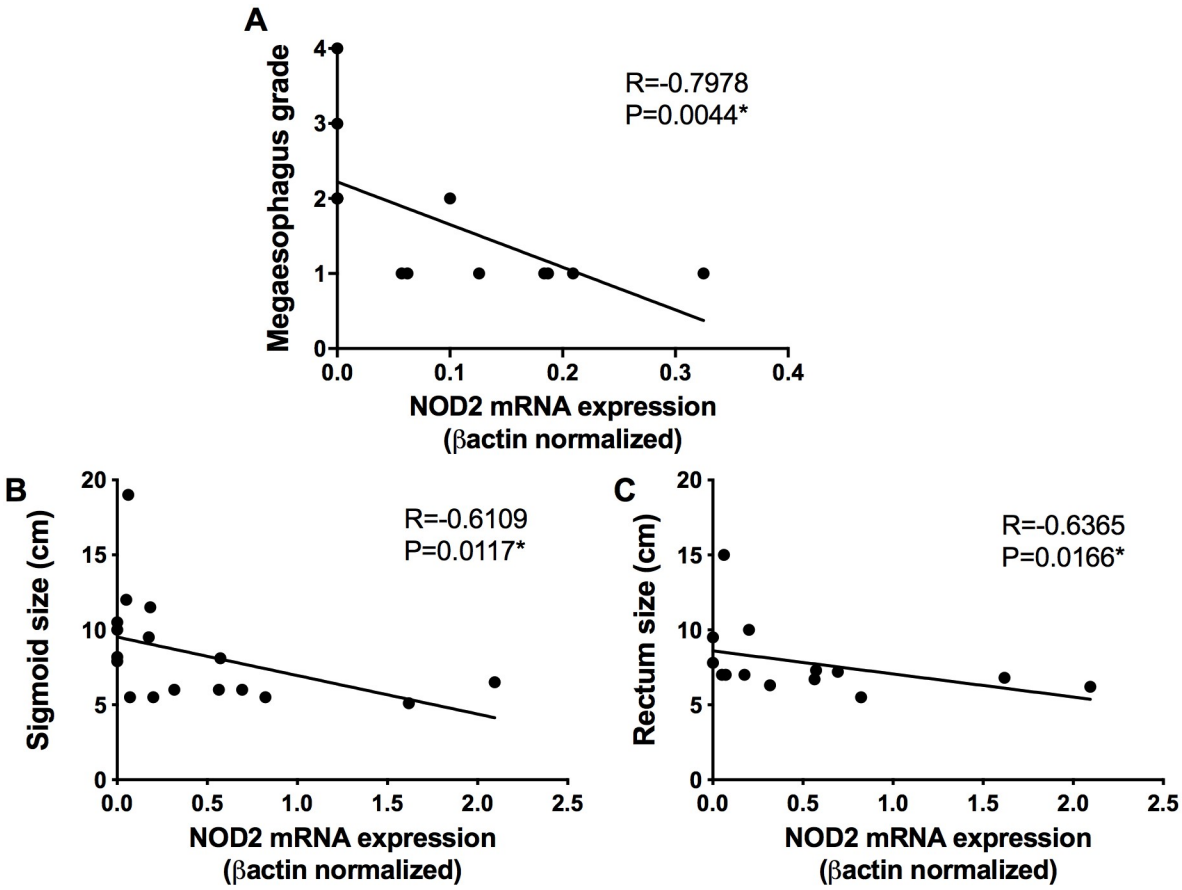

Fig 2. NOD2 expression is negatively correlated with the dilation degree of the esophagus, sigmoid and rectum. The mRNA expression levels of NOD2 were determined by real-time PCR in peripheral blood mononuclear cells of patients with the digestive and cardiodigestive clinical forms of Chagas'disease and correlated with megaesophagus grade (n = 13) **(A),** sigmoid size (n = 17) **(B)** and rectum size (n = 17) **(C).** The mRNA expression levels were normalized to the expression level of β-actin. Spearman test was used.

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decreased intestinal motility at 15, 180 and 360 days after infection, when compared to C57BL/6 animals (Fig 3B), indicating intestinal alterations.

In attempt to evaluate the tissue parasitism, inflammation and hypertrophy of the gastrointestinal tract, histopathological analysis was performed in WT and NOD2$^{-/-}$ in the acute (19 days) and chronic phases (12 months) of infection (Figs 4A–4N, 5A and 5B). The infection in the NOD2$^{-/-}$ animals caused intense focal inflammation in the colon during the chronic phase (Fig 4E, 4F and 4M), when compared to C57BL/6 mice (Fig 4B, 4C and 4M). Jejunum was the most affected region of the intestine, with intense inflammation in the NOD2$^{-/-}$ mice (Fig 4K, 4L and 4N) compared to C57BL/6 mice (Fig 4H, 4I and 4N). The inflammatory infiltrate presents a predominance of mononuclear cells, reaching the entire thickness of the muscular layers, sometimes forming cellular cords between the muscular layers and covering the ganglionic elements of the enteric nervous system. The colon of the NOD2$^{-/-}$ and C57BL/6 infected animals presented increased thickness of the muscularis propria, when compared to uninfected animals. However, NOD2$^{-/-}$ infected mice showed a more marked increase in the colon thickness of the muscularis propria than C57BL/6 infected animals (Fig 4C, 4F and 4M). Uninfected NOD2$^{-/-}$ mice presented a discrete increase in colonic wall thickness when compared to uninfected C57BL/6 animals (Fig 4M). The mice infection with a parasite isolated from a

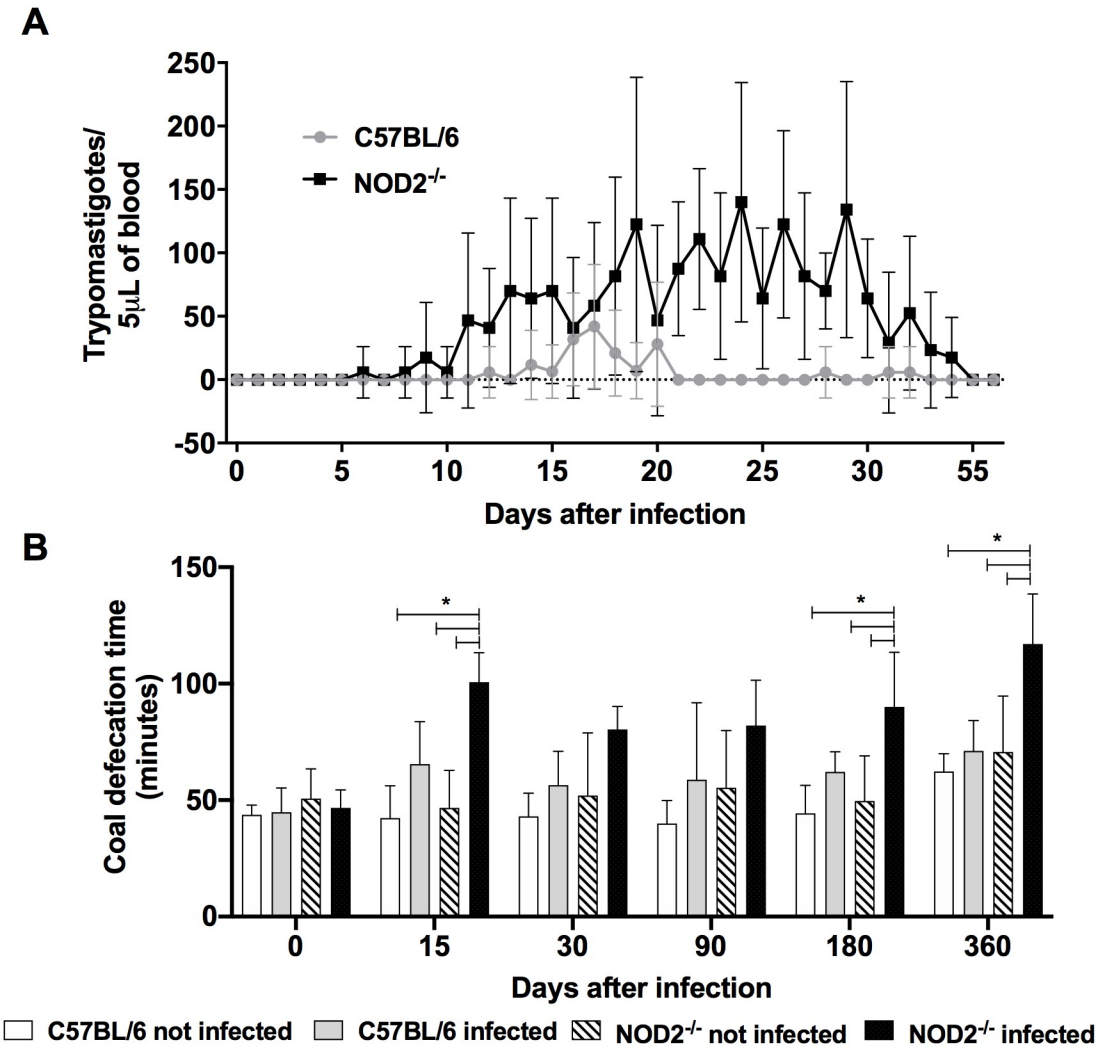

Fig 3. NOD2$^{-/-}$ mice infected with *Trypanosoma cruzi* showed reduced intestinal motility in acute and chronic phases of infection. Parasitemia (A) and survival (B) of female, C57BL/6 and NOD2$^{-/-}$ mice infected with the intraperitoneal route with 10^3 trypomastigote blood forms of RN25 isolate (Tc-II) of *Trypanosoma cruzi*. Gastrointestinal motility was evaluated by the mean time of elimination of activated coal in infected and non-infected C57BL/6 and NOD2$^{-/-}$ mice. The data are representative of 2 independent experiments with n = 10; * $p < 0.05$.

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patient with digestive form of the disease led to an increasing in the jejunum wall thickness in both NOD2$^{-/-}$ and C57BL/6 infected mice, when compared to uninfected controls (Fig 4N), but the jejunum wall thickness in NOD2$^{-/-}$ infected animals was bigger when compared with C57BL/6 infected mice (Fig 4I, 4L and 4N). C57BL/6 and NOD2$^{-/-}$ mice show similar parasitism during the acute and chronic phases of infection (Fig 5A). The parasitism was higher in acute than in chronic phase in both groups (Fig 5A). Semiquantitative analysis of the inflammatory foci in colon fragments of *T. cruzi*-infected mice demonstrated that NOD2 deficient animals present high inflammation in the colon during the acute and chronic phases, when compared to C57BL/6 animals (Fig 5B). These data emphasize the importance of NOD2 receptor in preventing the development of gastrointestinal tract alterations in *T. cruzi*-infected mice.

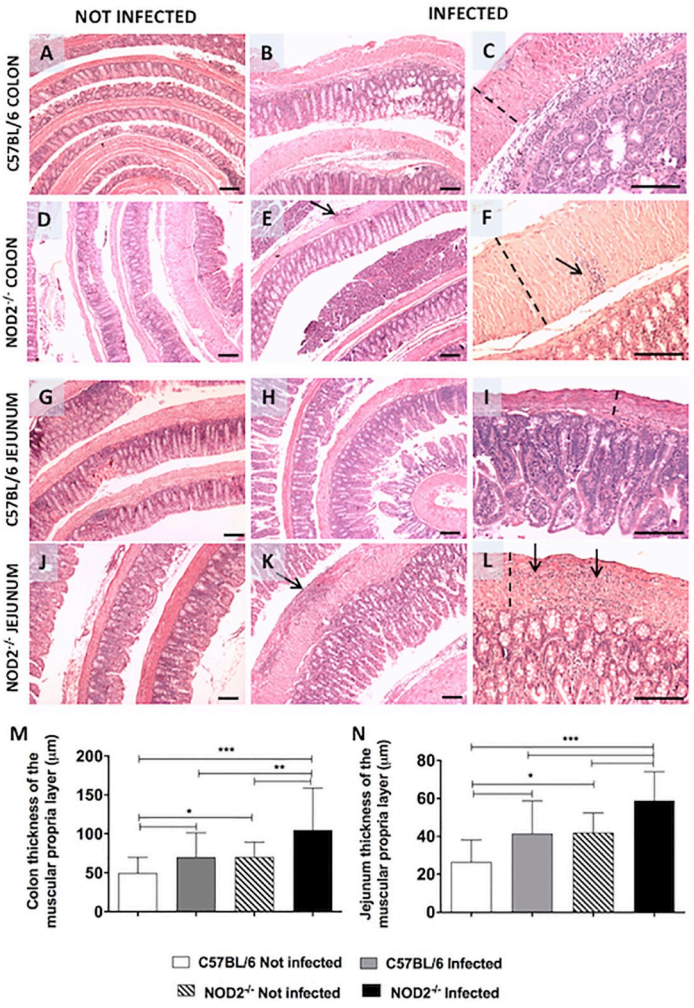

Fig 4. NOD2$^{-/-}$ animals show moderate inflammation and increased thickness of the longitudinal and circular muscular layer in the colon and jejunum during the chronic phase of the infection. Histopathology of colon (A, B, C, D, E, F) and jejunum (G, H, I, J, K, L) of C57BL/6 (A, B, C, G, H, I) and NOD2$^{-/-}$ (D, E, F, J, K, L) female mice not infected and intraperitoneally infected with 10^3 blood trypomastigotes forms of RN 25 (Tc-II) strain of *Trypanosoma cruzi* are pictured in 200× (left and middle column) and 400× (right column) magnifications. Quantification of the colon (M) and jejunal wall thickness (N) were performed in six animals of each group euthanized after 12 months. Scale bar = 50 μm. Arrows = inflammatory foci. Dotted lines indicate muscle wall thickness. The data are representative of two independent experiments. The bars graphs are plotted as mean ± SD $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

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Reduced intestinal motility and intestine hypertrophy of NOD2$^{-/-}$ is correlated to enhanced inflammatory markers in the colon during acute and chronic phases

The immunopathogenic mechanisms that lead to changes in the gastrointestinal tract, megacolon and megaesophagus formation depends on the presence of the parasite, inflammation and denervation. Thus, the expression of inflammatory markers that may contribute to this process was evaluated during the acute and chronic phases after *T. cruzi* infection. Acute phase evaluation showed increased expression of TLR-2, TLR-4, T-Bet, IFN-γ, iNOS, IL-10, TNF-α (Fig 6A–6G), and reduced IL-17 and defensin-A (Fig 6H and 6I) mRNA expression in the

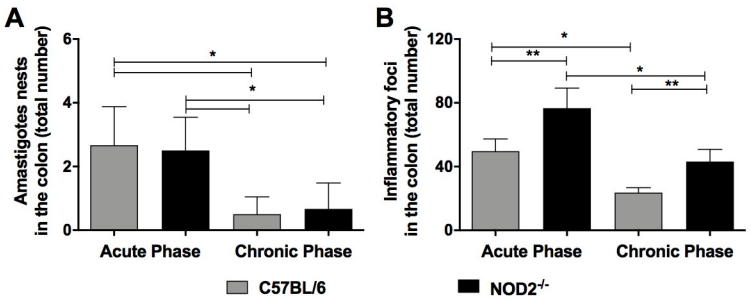

Fig 5. NOD2$^{-/-}$ animals showed similar parasitism and increased inflammation in the colon during the acute and chronic phases. Quantification of amastigote nests (A) and inflammatory foci (B) were performed by count 30 fields (200× magnification in optical microscope) from H&E slides of the colon of C57BL/6 and NOD2$^{-/-}$ mice infected. Acute (19 days) and chronic (12 months) phases are shown. n = 6 for each group. Data are representative of two independent experiments and are expressed as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$.

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colon of *T. cruzi* infected animals, compared to uninfected mice. Moreover, NOD2$^{-/-}$ infected mice showed higher TLR-2, TLR-4, T-Bet, IFN-γ and iNOS (Fig 6A–6E) than C57BL/6 animals. Similar levels of IL-17 and defensin-A mRNA expression was observed in NOD2$^{-/-}$ and C57BL/6 infected animals during acute phase of infection (Fig 6H and 6I). During the chronic phase we observed similar mRNA expression of TLR2 and IL-10 (Fig 7A and 7B) in the colon of NOD2$^{-/-}$ and C57BL/6 *T. cruzi*-infected mice. On the other hand, there was increased expression of TLR-4, T-Bet, TNF-α, IFN-γ, IL-17, iNOS, and defensin-A mRNA (Fig 7C–7I) in the colon of NOD2$^{-/-}$ when compared to WT mice. The higher expression of inflammatory mediators in NOD2 deficient mice and the decrease of IL-10 possibly contributes to the increase of musculature and appearance of lesions in the colon.

Discussion

We evaluated the role of NOD2 receptor in the lesion genesis of the gastrointestinal tract during the experimental infection by *T. cruzi* and its participation in the development of the digestive clinical form in patients with Chagas' disease. The results indicate that deficiency in the expression of the NOD2 receptor is correlated with the appearance of changes in the gastrointestinal tract during experimental infection in mice and also with the development of the digestive clinical form of Chagas' disease in patients.

We observed here that patients with digestive and cardiodigestive forms of Chagas' disease present absence or reduced NOD2 mRNA expression. We also demonstrated a negative correlation between the NOD2 mRNA expression with the degree of esophageal dilation, sigmoid and rectum sizes. These results indicate that NOD2 molecule has a protective role against the development of gastrointestinal tract lesions in patients with Chagas' disease. The NOD2 receptor is highly expressed in Paneth cells being responsible for the regulation of the intestinal microbiota through the production of antimicrobial components such as defensins [29, 59]. NOD2 deficient expression influences the composition of the microbiota because its absence leads to a higher number of pathogenic bacteria in the gastrointestinal tract [60] and is also involved with the development of inflammatory bowel diseases. Patients with Crohn's disease present alteration of the intestinal microbiota, with increase of *Bacteroidetes*, *Proteobacteria* and reduction of *Firmicutes* [61]. Patients with Chagas' disease with megaesophagus and megacolon present increased bacterial growth in the gastrointestinal tract when compared to the microbiota of healthy individuals [62, 63]. In addition, chagasic patients with the digestive

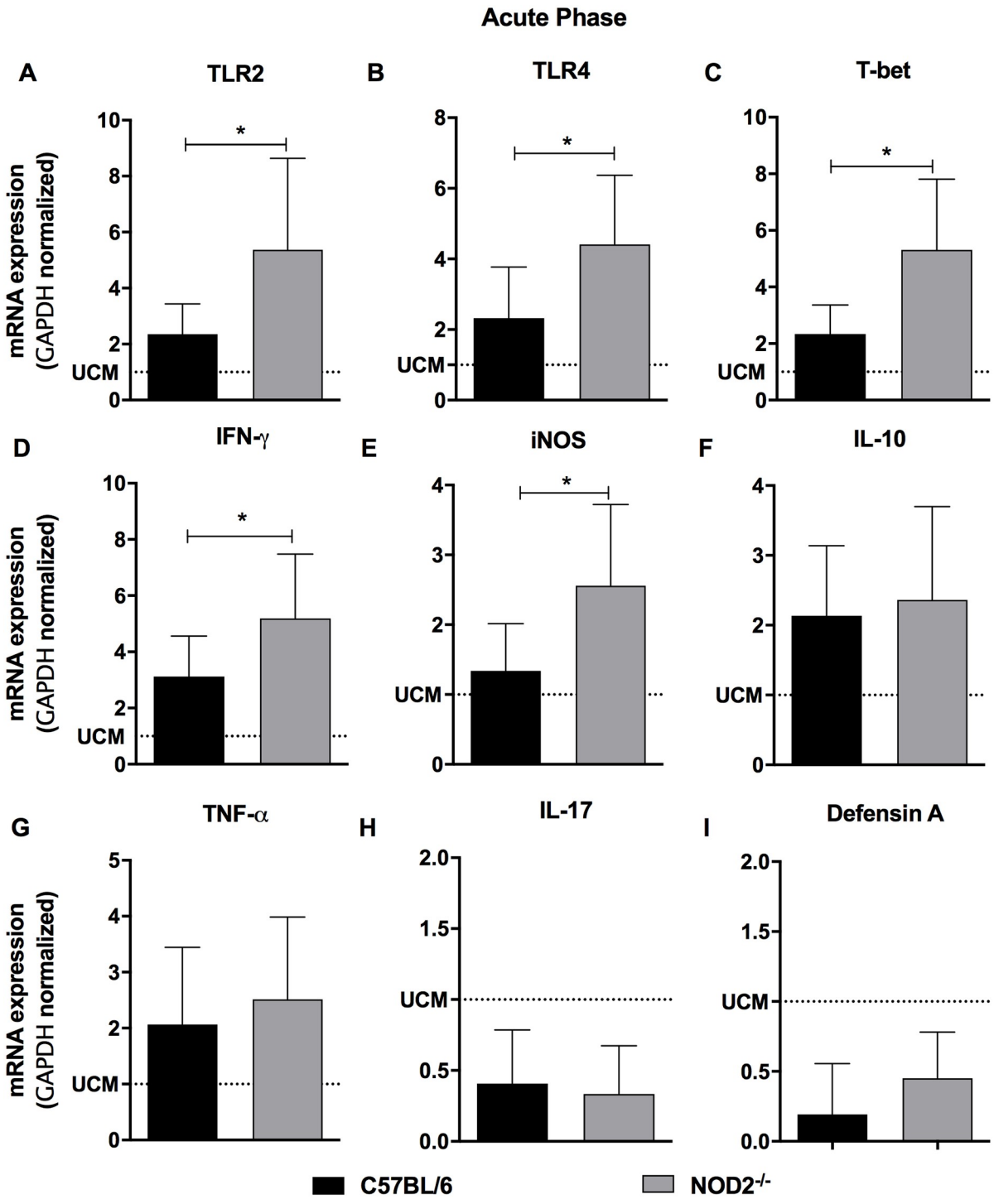

Fig 6. NOD2$^{-/-}$ mice presented high expression of inflammatory markers in the colon during the acute phase of *Trypanosoma cruzi* infection. The expression of TLR2 (A), TLR4 (B), T-bet (C), IFN-γ (D), iNOS (E), IL-10 (F), TNF-α (G), IL-17 (H) and defensin-A (I) were determined by real-time PCR in the colon of C57BL/6 and NOD2$^{-/-}$ infected mice and euthanized 19 days after infection. Data are representative of two independent experiments and expressed as mean ± SD. * $p < 0.05$. Dotted lines represent uninfected control mice (UCM). n = 6 for each group.

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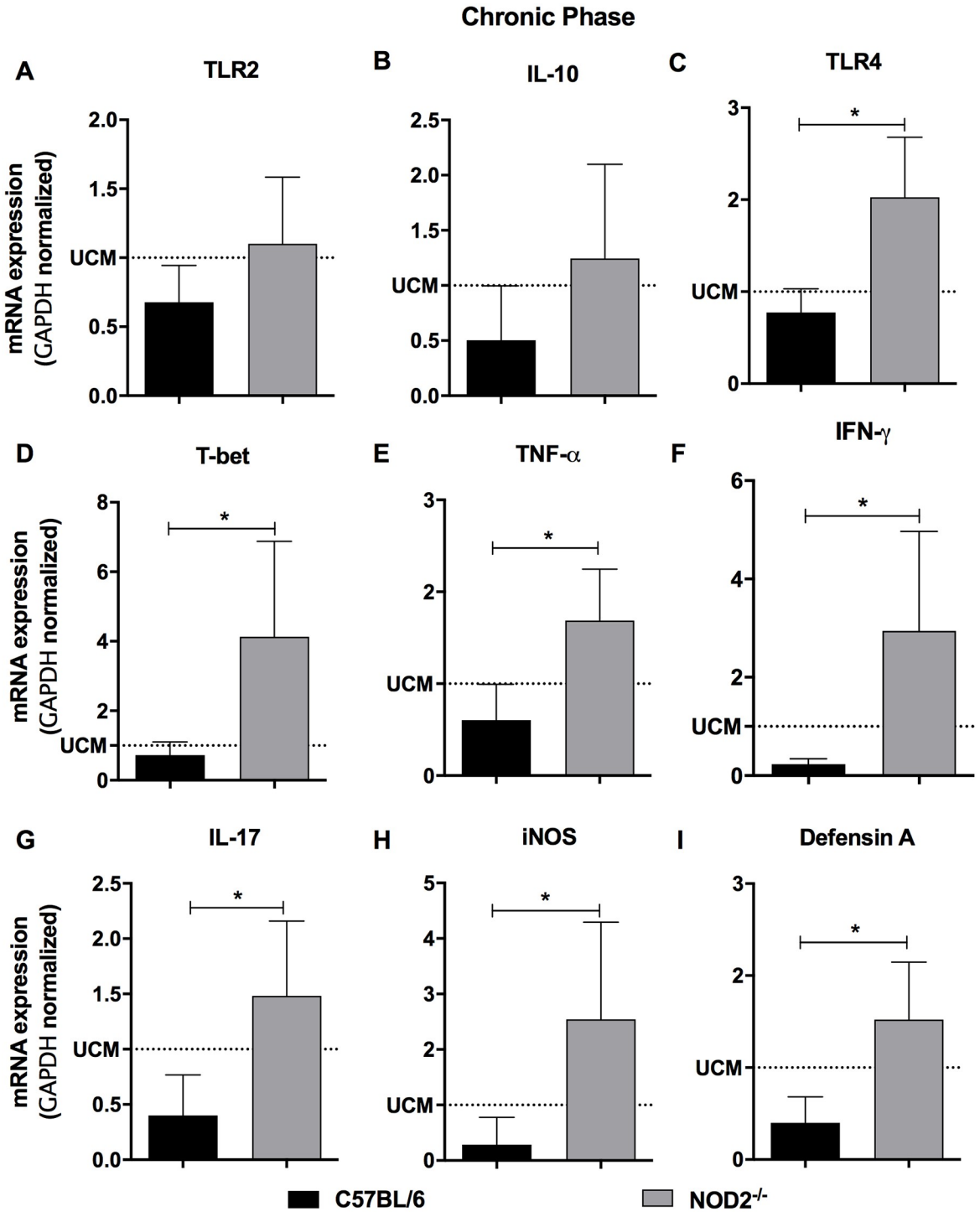

Fig 7. NOD2$^{-/-}$ mice presented high expression of inflammatory markers in the colon during the chronic phase of *Trypanosoma cruzi* infection. The expression of TLR2 (A), IL-10 (B), TLR4 (C), T-bet (D), TNF- (E), IFN-γ (F), IL-17 (G), iNOS (H) and defensin-A (I) were determined by real-time PCR in the colon of C57BL/6 and NOD2$^{-/-}$ infected mice and euthanized 12 months after infection. Data are representative of two independent experiments and expressed as mean ± SD. * $p < 0.05$. Dotted lines represent uninfected control mice (UCM). n = 6 for each group.

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form have higher number of bacteria with pathogenic potential, which is positively correlated with esophageal dilatation [63].

In the present study, increased expression of α-defensin 6 was observed in patients with the digestive form, compared to indeterminate and cardiac patients. Defensin production is stimulated after the contact of the bacteria with the digestive tract epithelium. Besides NOD2 pathway, the α-defensin production can also be induced through Toll-Like Receptors (TLR) [64–69] in Paneth Cells. In fact, we have previously demonstrated that those same patients have intact TLR family transcripts expression, being TLR8 overexpressed in digestive patients [28]. The microbiota dysbiosis observed in digestive chagasic patients [62, 63, 70] might be crucial for the elevated α-defensin levels in attempt to play its role through the formation of nanofibrils that protect intestinal mucosa from bacterial invasion [71–73]. Even in the absence or reduced expression of NOD2 in digestive and cardiodigestive patients, we showed that its adapter molecule RIP2 was overexpressed in those patients. This can be due a compensate mechanism or as consequence of NOD1 signaling activated in an attempt to induce cytokine and NO production to control the *T*. *cruzi* [74]. The increased expression of RIP2 in digestive and cardiodigestive patients can exacerbate the pathway to induce a greater cytokines production and, consequently, inflammation that contributes to the formation of the mega syndrome.

Another feature that can contribute to megacolon and megaesophagus formation is the parasite tropism to the gastrointestinal tract. Together with microbiota dysregulation and NOD2 deficiency, the presence of the parasites generates even more tissue damage and inflammation in the intestinal and esophageal epithelium, leading to denervation and contributing to organ pathology. Thus, our data suggest that NOD2 receptor plays an important role in the protection against lesions genesis of digestive tract in chagasic patients. Is important to note that, as described by others [7, 75–79], we also observed that the presence of megacolon is more frequent than the presence of megaesophagus in these chronic chagasic patients. This aspect is influenced by the anatomy of the organ, because in the esophagus, the alimentary content passes more easily influenced by gravity, whereas in the colon and rectum it presents a natural tendency of stagnation. Also, the colon and rectum present a higher number and diversity of bacteria than the esophagus [80], which could contribute to intensify inflammation, denervation and dilatation observed in the colon and rectum.

In order to confirm the importance of the NOD2 receptor in the genesis of digestive tract lesions in Chagas' disease, the development of gastrointestinal tract lesions was evaluated in NOD2 knockout mice infected with *T*. *cruzi* obtained from a digestive patient. Decreased intestinal motility was observed in NOD2$^{-/-}$ mice, indicating alterations in the gastrointestinal tract. The histopathological analysis of the colon demonstrated a moderate inflammatory lesion in the sub serous and muscular layer, more pronounced in NOD2$^{-/-}$ animals than C57BL/6 during the acute phase of infection. The lesions in the colon induced by *T*. *cruzi* during experimental infection in the acute phase include parasitism, degeneration and necrosis of muscle fibers. These are followed by intramuscular fibrosis, denervation and enlargement of the colon wall in the presence of sustained focal and moderate inflammation of the muscular propria by 15 months post infection in chronic phase [30]. NOD2$^{-/-}$ animals presented more intense focal inflammation in the colon and jejunum wall during acute and chronic phases of infection impacting in the thickness of the muscular layers and ganglionic elements of the enteric nervous system. These results indicate that infection with a parasite isolated from a patient with digestive form generates a progressive inflammation distributed in foci throughout the entire gastrointestinal tract, which altogether contribute significantly to the denervation process during the course of the disease.

The TLR2 and TLR4 activation by GPI-anchors and glycoinositolphospholipids derived from *T*. *cruzi*, respectively, leads NFκβ activation, IL-12, IFN-γ, TNF-α, IL-17 production and

iNOS activation [81–83]. Besides being important to kill the parasite, when in excess, TNF-α, IFN-γ and iNOS contribute to myenteric plexus degeneration during *T*. *cruzi* experimental infection [24]. Here, we demonstrated that NOD2-deficient mice infected with *T*. *cruzi*, which have an increased colon size, produce higher levels of these inflammatory mediators *in situ* when compared to WT animals. Interestingly, NOD2 is a negative regulator of TLR2 signaling, and then NOD2 deficiency can result in the production of Th1-related cytokines in environments rich in TLRs, such as the intestine [84]. In fact, we observed that NOD2 deficient mice showed high TLR2 and inflammatory mediators expression during the acute phase of the infection. These characteristics point to the central role of NOD2 receptor in the control of intestinal homeostasis, and could explain the increase in the long-term inflammation, which is the substrate of myenteric plexus denervation and exacerbated muscle hypertrophy generated by the *T*. *cruzi* strain with gastrointestinal tract tropism used in this investigation. Most likely both parasite tropism and chronic inflammation influence gastrointestinal tract injury.

Together, our results indicate that deficiency in NOD2 expression associated to *T*. *cruzi* strain that causes persistent intestinal inflammation promotes the breakdown of homeostasis of the digestive system, making patients more susceptible to the development of the digestive form of Chagas' disease. Thus, it is possible to hypothesize that the bacteria present in the microbiota are able to cross the mucus and IgA barriers, coming into contact with the epithelial layer of the digestive tract and inducing or potentiating the inflammation generated by the parasite in NOD2-deficient patients. The inflammation is responsible for denervation of the intestinal tract, leading to dysmotility, incoordination and gradual loss of peristalsis [14, 46]. The intestinal tract will dilate and luminal contents will initiate the compression of the viscera generating also ischemic pathologies that will amplify the process of formation of the megaesophagus and megacolon [4, 15, 20, 45, 85]. Finally, NOD2 is a protective factor against the development of digestive form of Chagas' disease by impacting in the control of parasite-induced inflammation and microbiota homeostasis.

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Funding acquisition: Rosa Maria Esteves Arantes, Dario Simões Zamboni, Egler Chiari, Antônia Cláudia Jácome da Câmara, Lúcia Maria da Cunha Galvão, Paulo Marcos Matta Guedes.

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Software: Nathalie de Sena Pereira, Mayra Fernanda Ricci, Paulo Marcos Matta Guedes.

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Validation: Antônia Cláudia Jácome da Câmara, Lúcia Maria da Cunha Galvão, Paulo Marcos Matta Guedes.

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References

1. WHO. Chagas disease (also known as American trypanosomiasis). World Health Organization. https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis). 2020.
2. Andrade Z. Patologia da doença de Chagas. In: Brener Z; Andrade ZA. Trypanosoma cruzi e doença de Chagas. Rio de Janeiro: Guanabara Koogan 2000: 31.
3. Rezende JMH. Forma digestiva da doença de Chagas. In: Brener Z; Andrade Z. Trypanosoma cruzi e doença de Chagas. Guanabara Koogan 2000: 47.
4. Rassi A Jr., Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010; 375(9723):1388–402. Epub 2010/04/20. https://doi.org/10.1016/S0140-6736(10)60061-X PMID: 20399979.
5. Dias JC, Ramos AN Jr., Gontijo ED, Luquetti A, Shikanai-Yasuda MA, Coura JR, et al. 2nd Brazilian Consensus on Chagas Disease, 2015. Rev Soc Bras Med Trop. 2016; 49Suppl 1(Suppl 1):3–60. Epub 2016/12/17. https://doi.org/10.1590/0037-8682-0505-2016 PMID: 27982292.
6. Breniere SF, Carrasco R, Revollo S, Aparicio G, Desjeux P, Tibayrenc M. Chagas' disease in Bolivia: clinical and epidemiological features and zymodeme variability of Trypanosoma cruzi strains isolated from patients. Am J Trop Med Hyg. 1989; 41(5):521–9. Epub 1989/11/01. https://doi.org/10.4269/ajtmh.1989.41.521 PMID: 2510524.
7. de Andrade CM, Camara AC, Nunes DF, Guedes PM, Pereira WO, Chiari E, et al. Chagas disease: morbidity profile in an endemic area of Northeastern Brazil. Rev Soc Bras Med Trop. 2015; 48(6):706–15. Epub 2015/12/18. https://doi.org/10.1590/0037-8682-0235-2015 PMID: 26676495.
8. Perez-Ayala A, Perez-Molina JA, Norman F, Navarro M, Monge-Maillo B, Diaz-Menendez M, et al. Chagas disease in Latin American migrants: a Spanish challenge. Clin Microbiol Infect. 2011; 17(7):1108–13. Epub 2010/11/16. https://doi.org/10.1111/j.1469-0691.2010.03423.x PMID: 21073628.
9. Salvador F, Trevino B, Sulleiro E, Pou D, Sanchez-Montalva A, Cabezos J, et al. Trypanosoma cruzi infection in a non-endemic country: epidemiological and clinical profile. Clin Microbiol Infect. 2014; 20(7):706–12. Epub 2013/12/18. https://doi.org/10.1111/1469-0691.12443 PMID: 24329884.
10. del Sanchez-Guillen MC, Lopez-Colombo A, Ordonez-Toquero G, Gomez-Albino I, Ramos-Jimenez J, Torres-Rasgado E, et al. Clinical forms of Trypanosoma cruzi infected individuals in the chronic phase of Chagas disease in Puebla Mexico. Mem Inst Oswaldo Cruz. 2006; 101(7):733–40. Epub 2006/12/13. https://doi.org/10.1590/s0074-02762006000700005 PMID: 17160280.
11. Sanchez-Montalva A, Moris M, Mego M, Salvador F, Accarino A, Ramirez K, et al. High Resolution Esophageal Manometry in Patients with Chagas Disease: A Cross-Sectional Evaluation. PLoS Negl Trop Dis. 2016; 10(2):e0004416. Epub 2016/02/06. https://doi.org/10.1371/journal.pntd.0004416 PMID: 26848957.
12. Munoz J, Gomez i Prat J, Gallego M, Gimeno F, Trevino B, Lopez-Chejade P, et al. Clinical profile of Trypanosoma cruzi infection in a non-endemic setting: immigration and Chagas disease in Barcelona (Spain). Acta Trop. 2009; 111(1):51–5. Epub 2009/05/12. https://doi.org/10.1016/j.actatropica.2009.02.005 PMID: 19426663.
13. Coura JR, Anunziato N, Willcox HP. [Chagas' disease morbidity. I—Study of cases originating in various states of Brazil, observed in Rio de Janeiro]. Mem Inst Oswaldo Cruz. 1983; 78(3):363–72. Epub 1983/07/01. https://doi.org/10.1590/s0074-02761983000300012 PMID: 6419008.
14. Koberle F, De AF. [Mechanism of destruction of the neurons of the peripheral nervous system in Changas' disease]. Hospital (Rio J). 1960; 57:1057–62. Epub 1960/06/01. PMID: 14410284.

15. Koeberle F. Enteromegaly and Cardiomegaly in Chagas Disease. Gut. 1963; 4:399–405. Epub 1963/12/01. https://doi.org/10.1136/gut.4.4.399 PMID: 14084752.

16. Koeberle G, Penha PD. [Chagas' mega-esophagus. (Quantitative studies on the intramural nervous-system of the esophagus)]. Z Tropenmed Parasitol. 1959; 10:291–5. Epub 1959/11/01. PMID: 14410383.

17. Jabari S, da Silveira AB, de Oliveira EC, Neto SG, Quint K, Neuhuber W, et al. Partial, selective survival of nitrergic neurons in chagasic megacolon. Histochem Cell Biol. 2011; 135(1):47–57. Epub 2010/12/25. https://doi.org/10.1007/s00418-010-0774-y PMID: 21184236.

18. Amorim MC-NA. A histopathologia e pathogenese do megaesôfago e megareto. Considerações em torno de um caso de "mal do engasgo". Anais da Faculdade de Medicina da Universidade de São Paulo. 1932; 8:27.

19. Koberle FNE. Etiologia e patogenia do megaesôfago no Brasil. Revista Paulista Medicina. 1955; 47:18.

20. Tafuri WL, Maria TA, Lopes ER. [Myenteric plexus lesions in the esophagus, jejunum and colon of chronic chagasic patients. Electron microscopy study]. Rev Inst Med Trop Sao Paulo. 1971; 13(2):76–91. Epub 1971/03/01. PMID: 5005737.

21. Gazzinelli RT, Oswald IP, Hieny S, James SL, Sher A. The microbicidal activity of interferon-gamma-treated macrophages against Trypanosoma cruzi involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-beta. Eur J Immunol. 1992; 22(10):2501–6. Epub 1992/10/01. https://doi.org/10.1002/eji.1830221006 PMID: 1396957.

22. Pinto NX, Torres-Hillera MA, Mendoza E, Leon-Sarmiento FE. Immune response, nitric oxide, autonomic dysfunction and stroke: a puzzling linkage on Trypanosoma cruzi infection. Med Hypotheses. 2002; 58(5):374–7. Epub 2002/06/12. https://doi.org/10.1054/mehy.2001.1401 PMID: 12056871.

23. Garcia SB, Paula JS, Giovannetti GS, Zenha F, Ramalho EM, Zucoloto S, et al. Nitric oxide is involved in the lesions of the peripheral autonomic neurons observed in the acute phase of experimental Trypanosoma cruzi infection. Exp Parasitol. 1999; 93(4):191–7. Epub 1999/12/22. https://doi.org/10.1006/expr.1999.4451 PMID: 10600444.

24. Arantes RM, Marche HH, Bahia MT, Cunha FQ, Rossi MA, Silva JS. Interferon-gamma-induced nitric oxide causes intrinsic intestinal denervation in Trypanosoma cruzi-infected mice. Am J Pathol. 2004; 164(4):1361–8. Epub 2004/03/25. https://doi.org/10.1016/s0002-9440(10)63222-1 PMID: 15039223.

25. Aliberti JC, Cardoso MA, Martins GA, Gazzinelli RT, Vieira LQ, Silva JS. Interleukin-12 mediates resistance to Trypanosoma cruzi in mice and is produced by murine macrophages in response to live trypomastigotes. Infect Immun. 1996; 64(6):1961–7. Epub 1996/06/01. https://doi.org/10.1128/IAI.64.6.1961-1967.1996 PMID: 8675294.

26. Teixeira ML, Rezende Filho J, Figueredo F, Teixeira AR. Chagas' disease: selective affinity and cytotoxicity of Trypanosoma cruzi-immune lymphocytes to parasympathetic ganglion cells. Mem Inst Oswaldo Cruz. 1980; 75(3–4):33–45. Epub 1980/07/01. https://doi.org/10.1590/s0074-02761980000200004 PMID: 6815409.

27. Ribeiro dos S, Hudson L. Denervation and the immune response in mice infected with Trypanosoma cruzi. Clin Exp Immunol. 1981; 44(2):349–54. Epub 1981/05/01. PMID: 6796312.

28. Pereira NS, Queiroga TBD, Nunes DF, Andrade CM, Nascimento MSL, Do-Valle-Matta MA, et al. Innate immune receptors over expression correlate with chronic chagasic cardiomyopathy and digestive damage in patients. PLoS Negl Trop Dis. 2018; 12(7):e0006589. Epub 2018/07/26. https://doi.org/10.1371/journal.pntd.0006589 PMID: 30044791.

29. Franchi L, Warner N, Viani K, Nunez G. Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev. 2009; 227(1):106–28. Epub 2009/01/06. https://doi.org/10.1111/j.1600-065X.2008.00734.x PMID: 19120480.

30. Campos CF, Cangussu SD, Duz AL, Cartelle CT, de Noviello ML, Veloso VM, et al. Enteric Neuronal Damage, Intramuscular Denervation and Smooth Muscle Phenotype Changes as Mechanisms of Chagasic Megacolon: Evidence from a Long-Term Murine Model of Trypanosoma cruzi Infection. PLoS One. 2016; 11(4):e0153038. Epub 2016/04/06. https://doi.org/10.1371/journal.pone.0153038 PMID: 27045678.

31. Saruta M, Targan SR, Mei L, Ippoliti AF, Taylor KD, Rotter JI. High-frequency haplotypes in the X chromosome locus TLR8 are associated with both CD and UC in females. Inflamm Bowel Dis. 2009; 15(3):321–7. Epub 2008/10/24. https://doi.org/10.1002/ibd.20754 PMID: 18942751.

32. Steenholdt C, Andresen L, Pedersen G, Hansen A, Brynskov J. Expression and function of toll-like receptor 8 and Tollip in colonic epithelial cells from patients with inflammatory bowel disease. Scand J Gastroenterol. 2009; 44(2):195–204. Epub 2008/11/06. https://doi.org/10.1080/00365520802495529 PMID: 18985539.

33. Sanchez-Munoz F, Fonseca-Camarillo G, Villeda-Ramirez MA, Miranda-Perez E, Mendivil EJ, Barreto-Zuniga R, et al. Transcript levels of Toll-Like Receptors 5, 8 and 9 correlate with inflammatory activity in

Ulcerative Colitis. BMC Gastroenterol. 2011; 11:138. Epub 2011/12/22. https://doi.org/10.1186/1471-230X-11-138 PMID: 22185629.

34. Ortiz-Fernandez L, Garcia-Lozano JR, Montes-Cano MA, Conde-Jaldon M, Leo E, Ortego-Centeno N, et al. Association of haplotypes of the TLR8 locus with susceptibility to Crohn's and Behcet's diseases. Clin Exp Rheumatol. 2015; 33(6 Suppl 94):S117–22. Epub 2015/10/22. PMID: 26486764.

35. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schaffeler E, Schlee M, et al. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut. 2004; 53(11):1658–64. Epub 2004/10/14. https://doi.org/10.1136/gut.2003.032805 PMID: 15479689.

36. Ganz T, Lehrer RI. Defensins. Curr Opin Immunol. 1994; 6(4):584–9. Epub 1994/08/01. https://doi.org/10.1016/0952-7915(94)90145-7 PMID: 7946046.

37. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 2001; 411(6837):603–6. Epub 2001/06/01. https://doi.org/10.1038/35079114 PMID: 11385577.

38. McDonald C, Inohara N, Nunez G. Peptidoglycan signaling in innate immunity and inflammatory disease. J Biol Chem. 2005; 280(21):20177–80. Epub 2005/04/02. https://doi.org/10.1074/jbc.R500001200 PMID: 15802263.

39. Hisamatsu T, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. Gastroenterology. 2003; 124 (4):993–1000. Epub 2003/04/03. https://doi.org/10.1053/gast.2003.50153 PMID: 12671896.

40. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A. 2009; 106(37):15813–8. Epub 2009/10/07. https://doi.org/10.1073/pnas.0907722106 PMID: 19805227.

41. de Souza PR, Guimaraes FR, Sales-Campos H, Bonfa G, Nardini V, Chica JEL, et al. Absence of NOD2 receptor predisposes to intestinal inflammation by a deregulation in the immune response in hosts that are unable to control gut dysbiosis. Immunobiology. 2018; 223(10):577–85. Epub 2018/07/26. https://doi.org/10.1016/j.imbio.2018.07.003 PMID: 30041769.

42. Barnich N, Darfeuille-Michaud A. Adherent-invasive Escherichia coli and Crohn's disease. Curr Opin Gastroenterol. 2007; 23(1):16–20. Epub 2006/11/30. https://doi.org/10.1097/MOG.0b013e3280105a38 PMID: 17133079.

43. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008; 134(2):577–94. Epub 2008/02/05. https://doi.org/10.1053/j.gastro.2007.11.059 PMID: 18242222.

44. Vago AR, Andrade LO, Leite AA, d'Avila Reis D, Macedo AM, Adad SJ, et al. Genetic characterization of Trypanosoma cruzi directly from tissues of patients with chronic Chagas disease: differential distribution of genetic types into diverse organs. Am J Pathol. 2000; 156(5):1805–9. Epub 2000/05/04. https://doi.org/10.1016/s0002-9440(10)65052-3 PMID: 10793092.

45. Adad SJ, Andrade DCS, Lopes ER, Chapadeiro E. Contribuição ao estudo da anatomia patológica do megaesôfago chagásico. Rev Inst Med Trop São Paulo 1991; 33:8.

46. Koberle F. Chagas' disease and Chagas' syndromes: the pathology of American trypanosomiasis. Adv Parasitol. 1968; 6:63–116. Epub 1968/01/01. https://doi.org/10.1016/s0065-308x(08)60472-8 PMID: 4239747.

47. Camargo ME. Fluorescent antibody test for the serodiagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of Trypanosoma cruzi in a slide test. Rev Inst Med Trop Sao Paulo. 1966; 8(5):227–35. Epub 1966/09/01. PMID: 4967348.

48. Umezawa ES, Nascimento MS, Kesper N, Jr., Coura JR, Borges-Pereira J, Junqueira AC, et al. Immunoblot assay using excreted-secreted antigens of Trypanosoma cruzi in serodiagnosis of congenital, acute, and chronic Chagas' disease. J Clin Microbiol. 1996; 34(9):2143–7. Epub 1996/09/01. https://doi.org/10.1128/JCM.34.9.2143-2147.1996 PMID: 8862574.

49. de RJ, Lauar KM, de OA. [Clinical and radiological aspects of aperistalsis of the esophagus]. Rev Bras Gastroenterol. 1960; 12:247–62. Epub 1960/09/01. PMID: 13741121.

50. Ximenes CA, Rezende JM, Moreira H, Vaz MGM. Técnica simplificada para o diagnóstico radiológico do Megacolon chagásico. Revista da Sociedade Brasileira de Medicina Tropical. 1984; 17(23).

51. Castro C, Hernandez EB, Rezende J, Prata A. [Radiological study on megacolon cases in an endemic area for Chagas disease]. Rev Soc Bras Med Trop. 2010; 43(5):562–6. Epub 2010/11/19. https://doi.org/10.1590/s0037-86822010000500018 PMID: 21085870.

52. Santana SO. Susceptibilidade do Triatoma brasiliensis (hemiptera: reduviidae triatominae) à infecção por isolados do Trypanosoma cruzi (kinetoplastida, trypanosomatidae) do Estado do Rio Grande do Norte. Dissertação de Mestrado Programa de Pós Graduação em Ciências Farmacêuticas, Universidade Federal do rio Grande do Norte. 2017:69.

53. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science. 2005; 307(5710):731–4. Epub 2005/02/05. https://doi.org/10.1126/science.1104911 PMID: 15692051.

54. Pizzi TAM; Christen R; Hoecker G; Neghme A Estudios sobre inmunobiología de la enfermedades parasitarias: I influencia de la constituicion genetica en la resistencia de las lauchas a la infeccion experimental pelo Trypanosoma cruzi. Bol Inf Parasit Chile. 1949; 4:3.

55. Brener Z. Atividade terapeutica do 5-nitro-furaldeido-semicarbazona (nitrofurazona) em esquemas de duração prolongada na infecção experimental de camundongo pelo Trypanosoma cruzi. Revista do Instituto de Medicina Tropical de São Paulo. 1961; 3(1):7.

56. Marona HR, Lucchesi MB. Protocol to refine intestinal motility test in mice. Lab Anim. 2004; 38(3):257–60. Epub 2004/06/23. https://doi.org/10.1258/002367704323133637 PMID: 15207036.

57. de Oliveira GM, de Melo Medeiros M, da Silva Batista W, Santana R, Araujo-Jorge TC, de Souza AP. Applicability of the use of charcoal for the evaluation of intestinal motility in a murine model of Trypanosoma cruzi infection. Parasitol Res. 2008; 102(4):747–50. Epub 2007/12/29. https://doi.org/10.1007/s00436-007-0829-8 PMID: 18163190.

58. Arantes RM, Nogueira AM. Distribution of enteroglucagon- and peptide YY-immunoreactive cells in the intestinal mucosa of germ-free and conventional mice. Cell Tissue Res. 1997; 290(1):61–9. Epub 1997/09/11. https://doi.org/10.1007/s004410050908 PMID: 9377643.

59. Biswas A, Petnicki-Ocwieja T, Kobayashi KS. Nod2: a key regulator linking microbiota to intestinal mucosal immunity. J Mol Med (Berl). 2012; 90(1):15–24. Epub 2011/08/24. https://doi.org/10.1007/s00109-011-0802-y PMID: 21861185.

60. Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, et al. Nod2 is essential for temporal development of intestinal microbial communities. Gut. 2011; 60(10):1354–62. Epub 2011/03/23. https://doi.org/10.1136/gut.2010.216259 PMID: 21421666.

61. Seksik P, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, Marteau P, et al. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut. 2003; 52(2):237–42. Epub 2003/01/14. https://doi.org/10.1136/gut.52.2.237 PMID: 12524406.

62. Guimaraes Quintanilha AG, Azevedo dos Santos MA, Avila-Campos MJ, Saad WA, Pinotti HW, Zilberstein B. Chagasic megacolon and proximal jejunum microbiota. Scand J Gastroenterol. 2000; 35(6):632–6. Epub 2000/07/27. https://doi.org/10.1080/003655200750023606 PMID: 10912664.

63. Pajecki D, Zilberstein B, dos Santos MA, Ubriaco JA, Quintanilha AG, Cecconello I, et al. Megaesophagus microbiota: a qualitative and quantitative analysis. J Gastrointest Surg. 2002; 6(5):723–9. Epub 2002/10/26. https://doi.org/10.1016/s1091-255x(02)00028-8 PMID: 12399062.

64. Santamaria MH, Perez Caballero E, Corral RS. Unmethylated CpG motifs in Toxoplasma gondii DNA induce TLR9- and IFN-beta-dependent expression of alpha-defensin-5 in intestinal epithelial cells. Parasitology. 2016; 143(1):60–8. Epub 2015/11/03. https://doi.org/10.1017/S0031182015001456 PMID: 26522645.

65. Foureau DM, Mielcarz DW, Menard LC, Schulthess J, Werts C, Vasseur V, et al. TLR9-dependent induction of intestinal alpha-defensins by Toxoplasma gondii. J Immunol. 2010; 184(12):7022–9. Epub 2010/05/22. https://doi.org/10.4049/jimmunol.0901642 PMID: 20488791.

66. Rumio C, Sommariva M, Sfondrini L, Palazzo M, Morelli D, Vigano L, et al. Induction of Paneth cell degranulation by orally administered Toll-like receptor ligands. J Cell Physiol. 2012; 227(3):1107–13. Epub 2011/05/14. https://doi.org/10.1002/jcp.22830 PMID: 21567398.

67. Muniz LR, Knosp C, Yeretssian G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. Front Immunol. 2012; 3:310. Epub 2012/10/23. https://doi.org/10.3389/fimmu.2012.00310 PMID: 23087688.

68. Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol. 2000; 1(2):113–8. Epub 2001/03/15. https://doi.org/10.1038/77783 PMID: 11248802.

69. Kinnebrew MA, Ubeda C, Zenewicz LA, Smith N, Flavell RA, Pamer EG. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. J Infect Dis. 2010; 201(4):534–43. Epub 2010/01/13. https://doi.org/10.1086/650203 PMID: 20064069.

70. Robello C, Maldonado DP, Hevia A, Hoashi M, Frattaroli P, Montacutti V, et al. The fecal, oral, and skin microbiota of children with Chagas disease treated with benznidazole. PLoS One. 2019; 14(2):e0212593. Epub 2019/02/27. https://doi.org/10.1371/journal.pone.0212593 PMID: 30807605; PubMed Central PMCID: PMC6391005.

71. Bevins CL. Innate immune functions of alpha-defensins in the small intestine. Dig Dis. 2013; 31(3–4):299–304. Epub 2013/11/20. https://doi.org/10.1159/000354681 PMID: 24246978.

72. Nakamura K, Sakuragi N, Takakuwa A, Ayabe T. Paneth cell alpha-defensins and enteric microbiota in health and disease. Biosci Microbiota Food Health. 2016; 35(2):57–67. Epub 2016/05/21. https://doi.org/10.12938/bmfh.2015-019 PMID: 27200259.

73. Chairatana P, Nolan EM. Human alpha-Defensin 6: A Small Peptide That Self-Assembles and Protects the Host by Entangling Microbes. Acc Chem Res. 2017; 50(4):960–7. Epub 2017/03/16. https://doi.org/10.1021/acs.accounts.6b00653 PMID: 28296382.

74. Silva GK, Gutierrez FR, Guedes PM, Horta CV, Cunha LD, Mineo TW, et al. Cutting edge: nucleotide-binding oligomerization domain 1-dependent responses account for murine resistance against Trypanosoma cruzi infection. J Immunol. 2010; 184(3):1148–52. Epub 2010/01/01. https://doi.org/10.4049/jimmunol.0902254 PMID: 20042586.

75. Lopes ER, Rocha A, Meneses AC, Lopes MA, Fatureto MC, Lopes GP, et al. [Prevalence of visceromegalies in necropsies carried out in Triangulo Mineiro from 1954 to 1988]. Rev Soc Bras Med Trop. 1989; 22(4):211–5. Epub 1989/10/01. https://doi.org/10.1590/s0037-86821989000400008 PMID: 2518668.

76. Atias A. [Gastrointestinal Chagas' disease in Chile]. Bol Chil Parasitol. 1969; 24(1):70–4. Epub 1969/01/01. PMID: 4983557.

77. Lacerna K. Compromiso digestivo en la enfermidad de Chagas. Gac Med Boliviana. 1991; 15:4.

78. Franca Rodriguez M. Enfermedad de Chagas y megacolon en Uruguay. Rev Uruguaya Pat Clin y Microbiol. 1971; 9:7.

79. Atias A. Enfermedad de Chagas digestiva em Chile. Experiencia de 20 anos. Bol Hosp S Juan de Dios. 1980; 27:7.

80. Sundin OH, Mendoza-Ladd A, Zeng M, Diaz-Arevalo D, Morales E, Fagan BM, et al. The human jejunum has an endogenous microbiota that differs from those in the oral cavity and colon. BMC Microbiol. 2017; 17(1):160. Epub 2017/07/19. https://doi.org/10.1186/s12866-017-1059-6 PMID: 28716079.

81. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procopio DO, et al. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 2001; 167(1):416–23. Epub 2001/06/22. https://doi.org/10.4049/jimmunol.167.1.416 PMID: 11418678.

82. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. J Immunol. 2006; 177(6):3515–9. Epub 2006/09/05. https://doi.org/10.4049/jimmunol.177.6.3515 PMID: 16951309.

83. Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, Golenbock DT, et al. Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to infection with T. cruzi. J Immunol. 2004; 173(9):5688–96. Epub 2004/10/21. https://doi.org/10.4049/jimmunol.173.9.5688 PMID: 15494520.

84. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol. 2004; 5(8):800–8. Epub 2004/06/29. https://doi.org/10.1038/ni1092 PMID: 15220916.

85. Tafuri WL, de Maria TA. [On the behavior of the neurosecretory vesicular component of the megaesophagus in human trypanosomiasis cruzi]. Rev Inst Med Trop Sao Paulo. 1970; 12(5):298–309. Epub 1970/09/01. PMID: 5499564.