Neurodegeneration and Increased Production of Nitrotyrosine, Nitric Oxide Synthase, IFN- γ and S100 β Protein in the Spinal Cord of IL-12p40-Deficient Mice Infected with *Trypanosoma cruzi*

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Key Words

Trypanosoma cruzi • Central nervous system • Nitric oxide • IL-12 • S100 β • IFN- γ

Abstract

Background/Aim: Chagas' disease is caused by *Trypanosoma cruzi* and occurs in most Latin American countries. The protozoan may colonize the central nervous system (CNS) of immune-compromised human hosts, thus causing neuronal disorders. Systemic control of the intracellular forms of the parasite greatly depends on the establishment of a TH1 response and subsequent nitric oxide (NO) release. At the CNS, it is known that low concentrations of NO promote neuronal survival and growth, while high concentrations exert toxic effects and neuron death. Accounting for NO production by astrocytes is the glia-derived factor S100 β , which is overproduced in some neurodegenerative diseases. In the current work, we studied the expression of NO, interferon (IFN)- γ and S100 β in the spinal cord tissue of IL-12p40KO mice infected with *T. cruzi*, a model of neurodegenerative process. **Methods:** IL-12p40KO and wild-type (WT) female mice in-

fectured with *T. cruzi* Sylvio X10/4 (10⁵ trypomastigotes, intraperitoneally) were euthanized when IL-12p40KO individuals presented limb paralysis. Spinal cord sections were submitted to immunohistochemical procedures for localization of neurofilament, laminin, nitrotyrosine, NO synthases (NOS), IFN- γ and S100 β . The total number of neurons was estimated by stereological analysis and the area and intensity of immunoreactivities were assessed by microdensitometric/morphometric image analysis. **Results:** No lesion was found in the spinal cord sections of WT mice, while morphological disarrangements, many inflammatory foci, enlarged vessels, amastigote nests and dying neurons were seen at various levels of IL-12p40KO spinal cord. Compared to WT mice, IL-12p40KO mice presented a decrement on total number of neurons (46.4%, $p < 0.05$) and showed increased values of immunoreactive area for nitrotyrosine (239%, $p < 0.01$) and NOS (544%, $p < 0.001$). Moreover, the intensity of nitrotyrosine (16%, $p < 0.01$), NOS (38%, $p < 0.05$) and S100 β (21%, $p < 0.001$) immunoreactivities were also augmented. No IFN- γ -labeled cells were seen in WT spinal cord tissue, contrary to IL-12p40KO tissue that displayed inflammatory infiltrating cells and also some parenchymal cells positively labeled.

Conclusion: We suggest that overproduction of NO may account for neuronal death at the spinal cord of *T. cruzi*-infected IL-12p40KO mice and that IFN- γ and S100 β may contribute to NOS activation in the absence of IL-12.

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Introduction

Infection by the protozoan *Trypanosoma cruzi* causes Chagas' disease, an important health problem in Latin America. The most conspicuous pathologies presented by the human hosts with the chronic form of the disease are cardiomyopathy and digestive megasyndromes [1, 2]. The cerebral form is an infrequent complication of the acute phase, already mentioned by Carlos Chagas in the original description of the disease [1]. In addition, it has been shown that chronic chagasic patients that become immunodeficient because of HIV infection or specific drug treatment to avoid allograft rejection may undergo disease reactivation at the central nervous system (CNS). Meningoencephalitis with headache, fever, neurological deficits, altered mental status, coma and death make the CNS form of the disease a serious health problem [2–7]. However, only few studies have focused on the involvement of nervous tissue in human or experimental Chagas' disease.

Combined strategies are developed by the immune system to control *T. cruzi* dissemination during the infection, including cytokine secretion, cellular cytotoxicity and specific antibody production [8–13]. Interleukin (IL)-12 has been shown to be an important cytokine involved in this process, by stimulating the production of interferon (IFN)- γ by natural killer (NK) and CD4 $^{+}$ T cells [14, 15]. In turn, IFN- γ activates macrophages to release nitric oxide (NO) which is able to kill the ingested intracellular parasites [16, 17].

Aside its antiparasitic effects, studies have shown a dual role of NO in the nervous tissue. NO from different cellular sources functions as a neurotransmitter and also promotes neuronal survival and growth under low concentrations [18, 19]. However, in high concentrations, it may exert toxic effects leading neurons to death [20, 21]. The 3 known isoforms of NO synthases (NOS), that is, the neuronal (nNOS), the endothelial (eNOS) and the inducible (iNOS) forms, are found in the nervous tissue. Differently from the former two, iNOS is activated by Ca $^{2+}$ -independent mechanisms and yields large amounts of NO after IFN- γ stimulus [22–25]. Of note, it was also demonstrated that astrocytic iNOS activity and subse-

quent NO production can also be induced by the glia-derived factor S100 β [26], independently on any previous cytokine stimulus.

Few studies have reported behavioral signs of neurodegeneration in immunocompromised murine models of *T. cruzi* infection [25, 27]. Recently, we showed that IL-12p40 knockout (KO) mice infected with the Sylvio X10/4 clone of *T. cruzi* presented an ascending progressive paralysis, from the tail to the forelimbs [27]. These mice lack the shared p40 subunit of IL-12 and IL-23, the latter a recently described cytokine [28] which is important for proliferation [29] and maintenance of the inflammatory TH17 cells, a subset of CD4 $^{+}$ lymphocytes [30, 31]. In the present paper, we evaluated neuronal loss and the presence of NO, IFN- γ and S100 β in the spinal cord of *T. cruzi*-infected IL-12p40KO and wild-type (WT) mice during the phase of established motor impairments.

Material and Methods

Animals, Parasites and Infection

Eight- to ten-week-old female C57BL/6 WT mice, as well as mice deficient in IL-12/IL-23 (IL-12p40KO) in the C57BL/6 background, were bred in our isogenic mice facilities, Biomedical Sciences Institute, University of São Paulo, under pathogen-free conditions. They were kept under controlled temperature and humidity in a light and dark cycle of 12 h each and fed ad libitum. Food and water were placed on the bottom of the cage when mice developed neurological deficits. All experiments were carried out in accordance with the ethical guidelines for experiments with mice, the protocols being approved by the Health Animal Committee of the University of São Paulo.

Trypomastigote forms of *T. cruzi* of the Sylvio X10/4 clone [32] were obtained from infection of LLCMK2 cells. Nine mice of each group were infected intraperitoneally with 10 5 parasites and daily evaluated for their neurological and general conditions. Moreover, parasitemias were evaluated twice a week by microscopic examination of 5 μ l of tail vein-obtained blood samples, as previously described [33].

Euthanasia

When paralysis affected the forelimb movements and an IL-12p40KO mouse showed no conditions of living by itself (from days 45 to 58 after infection), it was deeply anesthetized with ketamin and xilazin (Vetbrands) and euthanized by transcardiac perfusion with 10 ml of isotonic saline at room temperature, followed by 50 ml of fixative solution of 4% paraformaldehyde (w/v; Merck) in 0.1 M phosphate-buffered saline (PBS), pH 6.9, for 6 min. For each knockout mouse, a paired asymptomatic infected WT control mouse was also euthanized, as above, on the same day after infection.

Tissue Preparation, Sectioning and Sampling

After perfusion, the entire spinal cord was removed, postfixed in 4% paraformaldehyde solution at 4°C, for 90 min, and then

rinsed in 10% sucrose (Merck) dissolved in 0.1 M PBS, pH 7.4, at 4°C, for 48 h. Spinal cords were reduced into 3 segments that contained the cervical, thoracic and lumbar regions, and oriented along the rostrocaudal axis. Sets of segments were imbedded in tissue-freezing medium (Jung), frozen in dry ice-cooled isopentane (Sigma) at -45°C, and stored at -70°C until use.

Transversal adjacent frozen sections (14 µm thick) were obtained in a cryostat (CM 3000; Leica) throughout the entire 3 segments and sampled systematically in such a manner that the entire spinal cord was sampled throughout 9 distinct points. For each IL-12p40KO mouse section, 1 paired-WT mouse section obtained from the same spinal cord level was placed beside it. Glass slides were stored at -70°C until use.

Immunohistochemistry Procedures for Protein Localization

Sections from WT and IL-12p40KO mouse spinal cords were washed 2× for 10 min in PBS 0.1 M, incubated with 5% fat-free milk solution in PBS for 1 h at room temperature, washed 2× for 10 min in PBS, and incubated with 0.05% H₂O₂ in PBS (v/v; Sigma) for 30 min, in order to quench endogenous peroxidase. Sections were washed again 2× for 10 min in PBS and then incubated overnight at 4°C with one of the following epitope-specific antibodies: rabbit anti-cow S100β protein (Dako) in a 1:1,500 dilution; rabbit anti-neurofilament-200 (NF-200; Sigma) in a 1:600 dilution; rabbit anti-laminin (Sigma) in a 1:200 dilution; rabbit anti-nitrotyrosine (Sigma) in a 1:500 dilution; rabbit anti-universal NOS (uNOS; Sigma) in a 1:100 dilution; biotin-labeled monoclonal rat anti-mouse IFN-γ (BD Pharmingen) in a 1:50 dilution. A rat polyclonal anti-*T. cruzi* Sylvio X10/4 serum, obtained from a chronically infected rat and pre-absorbed in a mixture of mouse spinal cord tissue and spleen cells to remove nonspecific antibodies, was used in a 1:70 dilution to visualize the parasite. Antibodies were diluted in PBS containing 0.5% Triton X-100 (Sigma) and 1% bovine serum albumin (Sigma). After 2 × 10 min washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG (Vector) or biotinylated goat anti-rat Ig (Pharmingen), diluted 1:250 as above, for 1 h at room temperature. Sections submitted to the biotinylated rat anti-mouse IFN-γ were not incubated with the secondary antibody. Sections were rinsed 2× for 10 min in PBS and incubated with avidin and biotin peroxidase complex (both 1:125; Vector) for 45 min at room temperature, washed 2 × 10 min in PBS and 1 × 10 min in Tris buffer 0.05 M (Sigma), pH 7.4. Visualization of immunoreactivity was achieved with 3-3'-diaminobenzidine tetrahydrochloride (0.03%, w/v; Sigma) as a chromogen and H₂O₂ (0.05%, v/v; Sigma), both diluted in Tris. The reaction was interrupted in Tris solution when the darkest elements in the sections were below saturation, which occurred generally after 3 min.

Stereological Analysis

Spinal cord sections obtained from WT and IL-12p40KO mice (9 sections per group, n = 3 mice for each group) were immunolabeled for NF-200, counterstained by Giemsa and submitted to the optical fractionator method to estimate the total number of neuron body profiles in the entire spinal cord, indistinctly on the gray and white matters, including the lesion sites. Every 630th section was systematically sampled ($f_1 = 630$) and analyzed using a Computer Assisted Stereological Toolbox (CAST) system, as previously detailed [34, 35]. In brief, a microscope was interfaced with a computer by a color video camera, the GRID software

package controlled the motorized X-Y stage and a microcator, also linked to the microscope, monitored the movements in a vertical direction (Z). The border of the entire section was outlined, the step rates were entered (500 µm in the X and Y directions) and the program created a series of uniformly sample fields throughout the section. A 100× oil-immersion objective was used to count the cells (Q^-) in a counting frame ($2,311 \mu\text{m}^2$, a_{frame}) created by the software. By knowing the step rates it was possible to calculate the second sampled fraction (f_2): $[(X \text{ step-length} \cdot Y \text{ step-length}) / a_{\text{frame}}]$. The sampling volume (dissector) on the Z-axis extended 8 µm deep (height of the dissector) after excluding the parts of the section close to the slide and cover sleep. The total thickness of the section was also measured, giving the third sampling fraction (f_3): height of the section/height of the dissector. After counting all NF-200 profiles, we estimated the total number of neurons in the sampled region of the spinal cord, from cervical to sacral levels, as calculated ($N_{\text{total}} = \Sigma Q^- \cdot f_1 \cdot f_2 \cdot f_3$).

Semi-Quantitative Microdensitometric/Morphometric Image Analysis

The S100β, nitrotyrosine, uNOS and IFN-γ immunoreactivities were measured on all sampled spinal cord sections (3 sections for each segment, totalizing 9 sections per spinal cord) of 3 WT and 3 IL-12p40KO mice by means of microdensitometric/morphometric image analysis using a Kontron-Zeiss KS400 image analyzer. For WT mice, which possessed preserved spinal cord tissue, fields of measurement were sampled in the anterior and posterior horns of the gray matter. For IL-12p40KO mice, we chose fields bordering the region of infiltrating cells for measurement of S100β, nitrotyrosine and uNOS. In the case of IFN-γ, we chose the inflammatory focus regions because most labeling was predominantly inside them. In the absence of inflammatory foci, fields of measurements were sampled in the anterior and posterior horns. The procedures have been described previously [36–39]. Briefly, a television camera acquired images from the microscope (×40 objective). After shading correction, a discrimination procedure was performed as follows: the mean gray values (MGV) and standard error mean (SEM) were measured in the above-described sampled fields and also in corresponding spinal cord areas devoid of specific labeling (background). The specific MGV was then defined as the difference between the background MGV value and the MGV of the discriminated profiles in sampled fields of $44.1 \times 10^{-3} \text{ mm}^2$. This parameter reflects the immunoreactive intensity in the discriminated profile and indicates the amount of the measured immunoreactivity per profile. The glass value was kept constant at 200 MGV. The procedure was repeated for each section to correct every specific labeling measurement for background. In the morphometric evaluation, the area of all immunoreactive discriminated profiles, including cytoplasm and processes (when present and labeled) was measured. This parameter was also used in the present analysis because it reflects the state of cell reaction and the amount of cells involved in a specific event.

Statistical Analysis

For statistical comparisons of stereology, immunoreactivity area and specific MGV data of immune-deficient mice and WT controls, the unpaired t test was applied by means of the PRISM 4 software (Graph-Pad Software).

Results

Spinal Cord Lesions in T. cruzi-infected IL-12p40KO Mice

Sylvio X10/4 clone of *T. cruzi* is a low virulence strain that does not cause patent parasitemia in WT mice [27, 40]. Similarly to this, we observed null parasitemia rates for infected IL-12p40KO mice along the experimental period. Meanwhile, after day 35 after infection, Sylvio X10/4-infected IL-12p40KO mice, but not WT mice, started developing a progressive ascending paralysis that culminated in complete forelimbs palsy and death. In order to analyze the elements involved in paralysis, paired spinal cords of Sylvio X10/4-infected IL-12p40KO and WT mice were studied at the histological level. No spinal cord lesion was observed in any of the infected WT mice (fig. 1a, b), while in infected IL-12p40KO mice, inflammatory foci were found along the entire rostrocaudal spinal cord, compromising both the white and gray matters (fig. 1c). Inflammatory foci were characterized by morphological tissue disarrangement and strong local infiltration of mono- and polymorphonuclear immune cells. Several dilated vessels were also visualized inside the larger lesion areas (fig. 1c, inset). Moreover, few small remaining neuronal soma processes were observed among infiltrating cells (fig. 1d). Unbiased stereological analysis showed a decrement on total number of neurons at the spinal cord of IL-12p40KO mice ($403,200 \pm 93,980$) compared to WT mice ($752,600 \pm 75,150$; $p < 0.05\%$). In addition, amastigote and tripomastigote forms of *T. cruzi* were found in the spinal cord tissue of IL-12p40KO mice only (fig. 1e, f).

Immunoreactivity Profiles

Nitrotyrosine immunoreactive profiles were seen in the neuronal cytoplasm and neuropil throughout the gray and white matter regions of both WT and IL-12p40KO mouse spinal cords (fig. 2a, b). Besides the similarities, labeling profiles were stronger in IL-12p40KO than in WT spinal cord tissue. Moreover, cells infiltrating the lesion regions were also labeled and vessels were clearly demarked in IL-12p40KO sections (fig. 2b). According to the semi-quantitative microdensitometric/morphometric image analysis of spinal cord sections, a significant increase in the nitrotyrosine immunoreactive area of discriminated cell profiles was observed in the spinal cord of IL-12p40KO mice compared to WT mice (fig. 3a, b; table 1). Moreover, discriminated cells of the IL-12p40KO spinal cords also showed higher concentration of nitrotyrosine immunoreactivity, evidenced

by the specific mean gray value measurements (fig. 3c, d; table 1).

As described for nitrotyrosine immunoreactivity, uNOS labeling cell profiles were found all over the spinal cord gray and white matter regions of WT and IL-12p40KO mice (fig. 2c, d). Even so, as for nitrotyrosine, stronger uNOS immunoreactivity was seen in IL-12p40KO spinal cord tissue, although vascular endothelial cells were not highly immunopositive (fig. 2d). The microdensitometric/morphometric image analysis showed increases in the area and intensity (specific MGv) of uNOS immunoreactivity profile in the measured regions of the IL-12p40KO spinal cord sections compared to WT ones (fig. 4; table 1).

No labeling for IFN- γ was seen in WT spinal cord (fig. 2e). With regard to IFN- γ immunoreactivity in IL-12p40KO sections, lesion areas were predominantly labeled, displaying small rounded immune-positive cells (fig. 2f). Moreover, in areas of preserved tissue surrounding the site of injury, positive-labeled nonspherical cells were also observed (fig. 2f, inset). In figure 5 we show the values of immunoreactive area and labeling intensity for IFN- γ on lesion regions of IL-12p40KO spinal cord. Due to the absence of IFN- γ in WT sections, it was not possible to quantify its immunoreactivity in this group.

Fig. 1. Spinal cord neurodegenerative aspects induced by *T. cruzi* infection. WT and IL-12p40KO mice were infected intraperitoneally with 10^5 *T. cruzi* Sylvio X10/4 clone tripomastigote forms, sacrificed when knockout mice presented complete limbs paralysis (45–58 days after infection) and the spinal cords ($n = 3$, each group) were submitted to immunohistochemical procedures. Representative spinal cord sections of infected WT mice, demonstrating preserved tissue morphology (**a**), intact vessels (**a**, inset, arrows) and healthy neurons (**b**, arrows). Representative spinal cord section of IL-12p40KO-infected mice, showing morphologically disrupted tissue (**c**) rich in inflammatory foci (bluish) and enlarged vessels (**c**, inset, arrows). **d** An amplified field of **c**, showing remaining small neuronal soma processes (arrows) among infiltrating cell nuclei. **e** IL-12p40KO mouse spinal cord section demonstrating tissue parasitism (brownish) along the entire lesion area. Proliferative *T. cruzi* amastigotes (**f**, left, arrows) and scarce infective trypomastigote forms (**f**, right, arrows) were seen in knockout mouse spinal cord. **a–d** Tissue immunolabeled for NF-200, a neuronal intermediate filament, and counterstained by Giemsa, evidencing neurons (brownish) and total cell nuclei (bluish), respectively. **a**, **c** Insets show sections immunolabeled for laminin, evidencing vessels. **e**, **f** Tissue immunolabeled for *T. cruzi* proteins (brownish) counterstained by Giemsa. **a**, **c**, **e** Scale bar = 200 μ m; **a**, **c** insets, scale bar = 100 μ m; **b**, **d** scale bar = 20 μ m; **f** scale bar = 10 μ m.

Table 1. Percentage of increase of immunoreactive area and labeling intensity (specific MGv) in *T. cruzi*-infected IL-12p40KO mouse spinal cord sections compared to WT tissue for different targets

		Area	Specific MGv
Nitrotyrosine	SC	239%, $p < 0.01$	16%, $p < 0.01$
	C	251%, $p < 0.05$	23%, $p < 0.05$
	T	146%, $p < 0.05$	12%, $p < 0.01$
	L	406%, $p < 0.01$	15%, $p < 0.05$
uNOS	SC	544%, $p < 0.001$	38%, $p < 0.05$
	C	432%, $p < 0.01$	48%, NS
	T	387%, $p < 0.001$	34%, $p < 0.05$
	L	1,060%, $p < 0.05$	33%, $p < 0.05$
S100 β	SC	17%, NS	21%, $p < 0.001$
	C	64%, NS	33%, $p < 0.05$
	T	24%, NS	10%, NS
	L	15%, NS	18%, NS

SC = Total spinal cord; C = cervical intumescences; T = thoracic region; L = lumbar intumescences; NS = not significant.

S100 β immunoreactivity was found in neuronal, glial and endothelial cells throughout the spinal cord gray and white matters of both WT (fig. 2g) and IL-12p40KO mice (fig. 2h). Glial cells possessing strong immunoreaction product were observed at the border of lesion areas in IL-12p40KO sections, meanwhile, no infiltrating S100 β immunopositive round shaped cell profiles were found (fig. 2h). S100 β immunoreactive area of discriminated profiles of IL-12p40KO and WT mice did not differ statistically one from the other (fig. 6a, b; table 1). However, labeling intensities of discriminated S100 β immunopositive cell profiles of the total spinal cord and cervical region were higher in IL-12p40KO mice than in WT mice (fig. 6c, d; table 1).

Discussion

The fact that IL-12p40KO mice cannot develop an efficient immune response is one factor that might have favored *T. cruzi* entrance in the spinal cord and subsequent tissue colonization. Moreover, akin to IL-12p40KO macrophages which show enhanced permissiveness to Sylvio X10/4 *T. cruzi* parasites [41], microglia and other CNS cells in IL-12p40KO mice might find difficulty on restraining intracellular parasite growth at the spinal

cord tissue. Persistence or even reinvasion by this protozoan might result in a chronic inflammatory process that, instead of resolving the local infection, could intensify tissue injury throughout release of pro-inflammatory and microbicidal substances which, in elevated concentrations, present tissue-damaging effects. In this context, we observed an elevated immunoreactivity for NO synthases and for nitrotyrosine, a metabolic product generated from the nitration of tyrosine residues by peroxynitrite, in spinal cord sections of infected IL-12p40KO mice. Increasing attention has been given to the dual role played by NO at the CNS, especially because of the differential effects it exerts at low and high concentrations, as those of prevention and induction of apoptosis by caspases regulation, respectively [42]. Because NO plays an important role on *T. cruzi* killing [43, 44], overproduction of this molecule could have accounted for the neurodegenerative process observed in the spinal cord of IL-12p40KO mice.

The control of *T. cruzi* dissemination largely depends on an effective TH1 response. During effector processes, antigen-presenting phagocytes engaged in bi-directional talk with TCR-engaged infiltrating T CD4+ or T CD8+ cells release IL-12 towards T cells, which, in turn, produce IFN- γ . This cytokine subsequently acts on phagocytes and induces iNOS to mediate the release of large amounts of NO, a potent tripanosomicide agent [14, 15, 25]. However, in spite of the important role played by IL-12 on IFN- γ induction [45–47], it has been reported that IL-12-deficient hosts manage to produce low amounts of IFN- γ in different settings [48–51]. In this context, IL-18 was shown to be an inducer of IFN- γ by T cells in IL-12KO mice infected with *T. cruzi* [48], largely contributing to NO production and subsequent protozoan dissemination control. In addition, others correlate enhanced IL-18-gene transcription to IFN- γ production during *T. cruzi* infection [47]. Of note, β -chemokines also contribute to NO production by mouse *T. cruzi*-infected macrophages

Fig. 2. Representative microphotographs showing *T. cruzi*-infected WT (a, c, e, g) and IL-12p40KO (b, d, f, h) mouse spinal cord immunoreactivity profiles of nitrotyrosine, for indirect NO visualization (a, b), of NO synthases (c, d), of IFN- γ (e, f) and of S100 β (g, h). For each IL-12p40KO mouse spinal cord, a paired WT spinal cord was submitted to the same immunostaining procedures. A nonimmune IFN- γ -positive cell is shown in f (inset). Arrows indicate neuron somas, arrowheads indicate vessels (clearly demarked in b) and asterisks show lesion areas. a–h Scale bars = 50 μ m; f inset, scale bar = 20 μ m.

Fig. 3. Nitrotyrosine immunoreactive area and intensity. After immunostaining procedures for nitrotyrosine, to indirectly visualize NO, 9 spinal cord sections of infected WT (n = 3) and IL-12p40KO mice (n = 3) were submitted to the microdensitometric/morphometric image analysis for measurement of area and intensity of the discriminated immunoreactivity profiles. Immunoreactive area of IL-12p40KO spinal cords was larger than that of WT ones for both total spinal cord (a) and its segments (b). Moreover, the intensity (specific MGv) of nitrotyrosine immunoreactivity in IL-12p40KO tissue was higher than in WT for the entire spinal cord (c) and its segments (d). □ = WT mice; ■ = IL-12p40KO mice; C = cervical intumescences; T = thoracic region; L = lumbar intumescences. Mean ± SEM. * p < 0.05; ** p < 0.01, according to unpaired t test.

Fig. 4. uNOS immunoreactive area and intensity. A universal antibody that binds the 3 NOS isoforms (neuronal, endothelial and inducible) was used. Nine spinal cord sections of infected WT (n = 3) and IL-12p40KO mice (n = 3) were submitted to the microdensitometric/morphometric image analysis for measurement of area and intensity of the discriminated immunoreactivity profiles. IL-12p40KO tissue sections presented increased uNOS immunoreactive area for the entire spinal cord (a) and its segments (b) when compared to the WT ones. Moreover, labeling intensity (specific MGv) presented by IL-12p40KO tissue was higher than that of WT spinal cord, in its totality (c) as well as in thoracic and lumbar segments (d). □ = WT mice; ■ = IL-12p40KO mice; C = cervical intumescences; T = thoracic region; L = lumbar intumescences. Mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001, according to unpaired t test.

[16]. By showing IFN- γ -immunolabeled cells at the spinal cord tissue of *T. cruzi*-infected IL-12p40KO mice, we corroborate our previous results, in which TCD8 cells and, at a lower level TCD4 cells isolated from the spinal cord of *T. cruzi*-infected IL-12p40KO mice were shown to produce IFN- γ [27]. Once IFN- γ immunoreactive profiles occurred predominantly in lesion areas, we suppose this

cytokine contributed to the production of NO, via iNOS, by activated macrophages/microglia. The role of IFN- γ in the nervous system has been object of various studies. IFN- γ has been pointed as an important mediator of neuronal plasticity, regulating synaptic plasticity [52], enhancing synaptogenesis [53] and neurogenesis [54], albeit it was also reported to inhibit dendritic growth and

Fig. 5. IFN- γ immunoreactive area and intensity. Nine spinal cord sections of infected WT (n = 3) and IL-12p40KO mice (n = 3) were submitted to the microdensitometric/morphometric image analysis for measurement of area and intensity of the discriminated immunoreactivity profiles. No immunoreactivity for IFN- γ was observed in infected WT spinal cord tissue. **a, b** IFN- γ immunoreactive area for IL-12p40KO total spinal cord and its segments, respectively. The labeling intensity (specific MGv) was also measured in IL-12p40KO entire spinal cord (**c**) and its segments (**d**). C = Cervical intumescences; T = thoracic region; L = lumbar intumescences.

Fig. 6. S100- β immunoreactive area and intensity. Nine spinal cord sections of infected WT (n = 3) and IL-12p40KO mice (n = 3) were submitted to the microdensitometric/morphometric image analysis for measurement of area and intensity of the discriminated immunoreactivity profiles. The immunoreactive area for S100 β did not statistically differ between WT and IL-12p40KO mice for the entire spinal cord (**a**) or its segments (**b**). Nonetheless, S100 β labeling intensity (specific MGv) was higher in IL-12p40KO spinal cord in its totality (**c**) and in the cervical intumescences (**d**), compared to the WT one. $\square$ = WT mice; $\blacksquare$ = IL-12p40KO mice; C = cervical intumescences; T = thoracic region; L = lumbar intumescences. Mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$, according to unpaired t test.

synapse formation [55]. Moreover, in addition to indirect neuronal damage caused by promotion of NO release in macrophages/microglia, Mizuno et al. [56] demonstrated that IFN- γ directly induces neurotoxicity by coupling with AMPA receptor GluR1 exclusively in neurons, eliciting Ca^{2+} influx and subsequent NO production via nNOS.

Besides nitrotyrosine labeling at the IL-12p40KO lesion areas, positive staining for this metabolite was also observed at the tissue bordering the lesion. Free diffusion of NO across membranes [for review, see 57] cannot satisfactorily explain the high nitrotyrosine immunoreactivity at the lesion-bordering tissue, since a similar labeling profile was also observed for NOS. In view of that, we

investigated other means of NOS activation independently on immune system cells. In this context, we focused on the glia protein S100 β , because in high concentrations this protein was shown to stimulate astrocytes to produce NO via iNOS [26] and, subsequently, cause neuronal cell death with apoptosis characteristics via a paracrine regulation [58]. According to our data, the increased immunoreactivity for S100 β at the IL-12p40KO spinal cord tissue suggests that this factor may have contributed to NO release, as stated by the nitrotyrosine immunoprofile.

Several studies have demonstrated NO involvement in neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, causing neurons death [59–62]. Of note, Arantes et al. [63] reported intrinsic intestinal denervation in *T. cruzi*-

infected mice, due to IFN- γ -induced NO. In line with this, in vitro neurodegeneration by IFN- γ -induced NO released from *T. cruzi*-infected peritoneal macrophages was also reported [64]. Likewise, here we suggest that infiltrating and resident glial cells stimulated by IFN- γ and S100 β were able to overproduce NO, which contributed to the neurodegenerative process seen in *T. cruzi*-infected IL-12p40KO spinal cord.

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