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## Different peripheral neuroendocrine responses to *Trypanosoma cruzi* infection in mice lacking adaptive immunity

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*Trypanosoma cruzi* infection in mice triggers neuroendocrine responses that affect the course of the disease. To analyze the contribution of adaptive immunity to these responses, comparative studies between normal C57Bl/6J and recombinase activator gene 1 (RAG-1)-deficient mice, which lack mature B and T lymphocytes, were performed. There was no difference between both types of mice in basal body weight. Following infection, higher parasitemia, increased IL-1 $\beta$  and IL-6 blood levels, less marked changes in lymphoid organs weight, no cardiomegaly, and earlier mortality were observed in RAG-1-deficient, compared with normal mice. The response of the hypothalamus-pituitary-adrenal axis after infection occurred earlier and was more intense in RAG-1-deficient mice than in normal mice. Noradrenaline concentration and serotonergic metabolism in the spleen, lymph nodes, and heart differed between RAG-1-deficient and normal mice. Our studies indicate that the absence of adaptive immunity to *T. cruzi* influences the neuroendocrine response to the infection with this parasite.

**Keywords:** recombinase activator gene 1-deficient mice; *Trypanosoma cruzi*; infection; corticosterone; noradrenaline; serotonin; sympathetic nervous system

### Introduction

Natural and adaptive immune responses can both trigger cytokine-mediated neuroendocrine responses capable of affecting the course of infectious diseases. For example, intense stimulation of the hypothalamus-pituitary-adrenal (HPA) axis is detected in mice during the course of acute infection with *Trypanosoma cruzi*.<sup>1</sup> The increase in glucocorticoid levels is responsible for the thymus atrophy observed during infection with this parasite, but is protective for the host. Indeed, there is an uncontrolled release of proinflammatory cytokines during the course of the infection and early mortality when the effect of the increased levels of corticosterone is abrogated by adrenalectomy or by administration of the glucocorticoid receptor blocker RU486.<sup>1</sup> This evidence indicates that coupled immune and

neuroendocrine responses are triggered during the infection with *T. cruzi*, the parasite that causes Chagas disease. This disease, also called American trypanosomiasis, is the fourth leading cause of death in Latin America. Although once confined to South and Central America, where it is endemic, it has now spread to other continents, particularly Europe. Indeed, over 120 million people are at risk of infection in 21 countries. In humans, the disease occurs in two stages: an acute phase, manifested shortly after infection, and a chronic state that may develop over 10 years. Chronic infection results in various neurological disorders, damage to the heart muscle (cardiomyopathy, the most serious manifestation), and sometimes dilation of the digestive tract (megacolon and megaesophagus). Left untreated, Chagas disease can be fatal, in most cases due to the cardiac sequelae.

The innate immune system has the ability to sense pathogens via germ line–encoded pattern recognition receptors. These receptors recognize pathogen associated molecular patterns (PAMPs), conserved molecules shared by several microorganisms, including *T. cruzi*, and trigger the activation of host innate responses.<sup>2–4</sup> Adaptive immunity to *T. cruzi* has been well characterized, with critical involvement of CD4<sup>+</sup> Th1 and CD8<sup>+</sup> T cells that recognize *T. cruzi*–specific antigens.<sup>2</sup> However, it is still unknown to what extent the absence of adaptive immunity influences neuroendocrine responses triggered by *T. cruzi* inoculation. To analyze whether combined T and B cell deficiency influences the response of the HPA axis and the sympathetic nervous system (SNS) and affects peripheral serotonergic mechanisms during *T. cruzi* infection, we performed comparative studies between normal C57Bl/6J (C57) mice and the recombinase activator gene 1 (RAG-1)–deficient mutant mice in the same genetic background. RAG-1–deficient mice lack mature B and T lymphocytes due to their incapacity to rearrange and recombine the immunoglobulin and T cell receptor genes.

## Material and methods

### *Mice and infection*

C57Bl/6J (C57) and RAG-1–deficient mice (original breeding pairs were obtained from Jackson Laboratory, Bar Harbor, ME) were bred in the animal facilities of the Universidad Abierta Interamericana, Rosario, Argentina. Mice were housed individually for 1 week before experiments were started and kept single-caged throughout the experiments in temperature-, humidity- and light (12-h cycles)–controlled rooms. One hundred trypomastigotes suspended in 100  $\mu$ L physiological saline were injected s.c. (50  $\mu$ L in each flank) when mice were 8–10 weeks old. The Tulahuén strain of *T. cruzi* used in this study was maintained by serial passages in C57Bl/6 suckling mice. Eighteen days postinfection (p.i.), groups of C57 and RAG-1–deficient mice were sacrificed, and blood obtained. The spleen, the inguinal lymph nodes, and the heart were collected and weighed. Results are expressed as relative to the body weight (organ weight/body weight  $\times$  100).

### *Survival*

A separate group of C57 and RAG-1–deficient mice was infected and left undisturbed. Survival was controlled daily.

### *Evaluation of parasitemia*

Bloodstream forms of *T. cruzi* were counted under standardized conditions by direct microscopic observation of 5  $\mu$ L heparinized blood obtained from the tip of the tail on day 17 p.i. Data are expressed as number of parasites/50 fields.

### *Corticosterone determination*

Plasma samples for hormone determinations were obtained from the tip of the tail under light ether narcosis between 8 and 10 a.m. before injection (time 0), and 7, 14, and 17 days p.i. Corticosterone levels in plasma were determined using a commercially available enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany).

### *Determination of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in plasma*

Blood samples were collected after sacrificing the animals 18 days p.i. Plasma was stored frozen at  $-20^{\circ}\text{C}$  until used for the determinations. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 concentrations were evaluated by ELISA, using commercially available kits (R&D), and the limit of detection was 5.1, 3, and 15.6 pg/mL, respectively. All samples were assayed in duplicate.

### *Neurotransmitter determination in the spleen, lymph nodes, and heart*

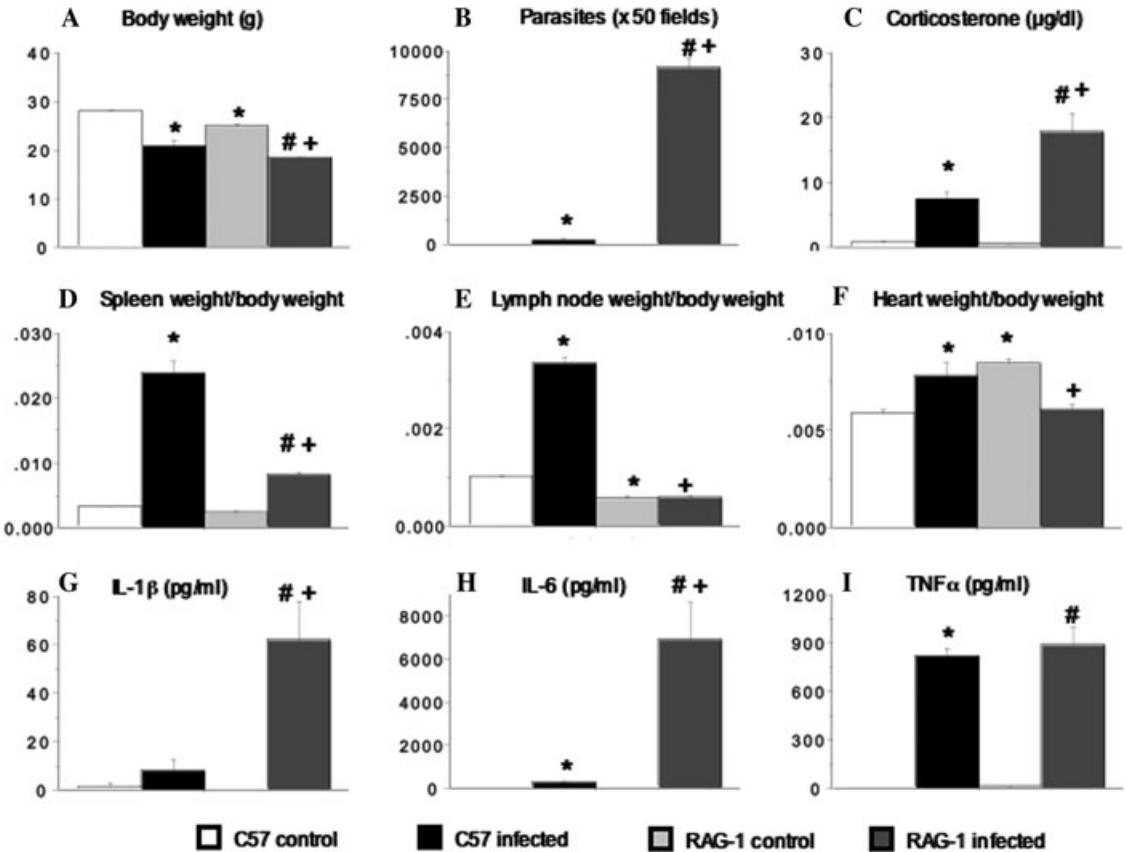
Noradrenalin (NA), 5-hydroxytryptamine (serotonin; 5-HT), its precursor tryptophan (Trp), and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined in the spleen, inguinal lymph nodes, and heart by HPLC with electrochemical detection, as described previously,<sup>5</sup> using the supernatant of tissue samples homogenized in 0.4 M HClO<sub>4</sub>. Results were expressed as concentration (ng neurotransmitter/g organ), and as the content of the neurotransmitter in the whole organ (ng neurotransmitter/g  $\times$  organ weight).

### *Statistical analysis*

Results are expressed as means  $\pm$  SEM. Data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's test for multiple comparisons or by nonparametric tests (Mann–Whitney *U* test for two samples and Kruskal–Wallis test for *k* samples).

## Results

Body weight, parasitemia, and plasma levels of some representative cytokines were evaluated in C57 and RAG-1–deficient mice 18 days after infection with



**Figure 1.** Body, spleen, lymph node, heart weight, parasitemia, corticosterone, and cytokine blood levels following *T. cruzi* infection. C57Bl/6J (C57) and RAG-1-deficient (RAG-1) mice were sacrificed 18 days after injection of 100 trypomastigotes (infected) or the vehicle alone (control). Organ weights are expressed as relative to body weight. Parasitemia and corticosterone plasma levels were evaluated on day 17 p.i. Results are expressed as mean ± SEM of determinations performed in 6–7 mice per group. \**P* < 0.05 versus C57 control; #*P* < 0.05 versus RAG-1-deficient control; +*P* < 0.05 versus C57 infected.

*T. cruzi* or inoculation of the vehicle alone. The body weight was decreased in both types of infected mice at this time (Fig. 1A). The spleen weight increased about fivefold following *T. cruzi* infection of C57 mice and only about threefold in RAG-1-deficient mice, and this difference was maintained when the weight of the organ was expressed relative to the body weight (Fig. 1D). The immune-deficient mice had smaller lymph nodes than C57 mice, and the weight did not increase after infection. In contrast, the weight of the lymph nodes of infected normal mice increased about threefold in infected C57 mice, also when the results were expressed relative to the body weight (Fig. 1E). The heart was chosen because it is a main target of the parasite.<sup>6</sup> Interestingly, while the heart of C57 mice increased

after infection, a reduction was noticed in RAG-1-deficient mice (Fig. 1F).

RAG-1-deficient mice had a parasite load that was 20-fold higher (Fig. 1B), and 10-fold higher IL-1β and IL-6 blood levels than C57 mice; however, although also increased as compared with the noninfected controls, no differences in TNF-α levels were detected (Fig. 1G, H, and I). Corticosterone blood levels on day 17 were also more than twofold higher in infected RAG-1-deficient as compared with infected C57 mice (Fig 1C). Blood levels of this hormone were also determined in these mice before infection (time 0), and 7 and 14 days later (Fig. 2A). No differences in basal levels were detected between noninfected, RAG-1-deficient and C57 mice. Corticosterone levels were already significantly

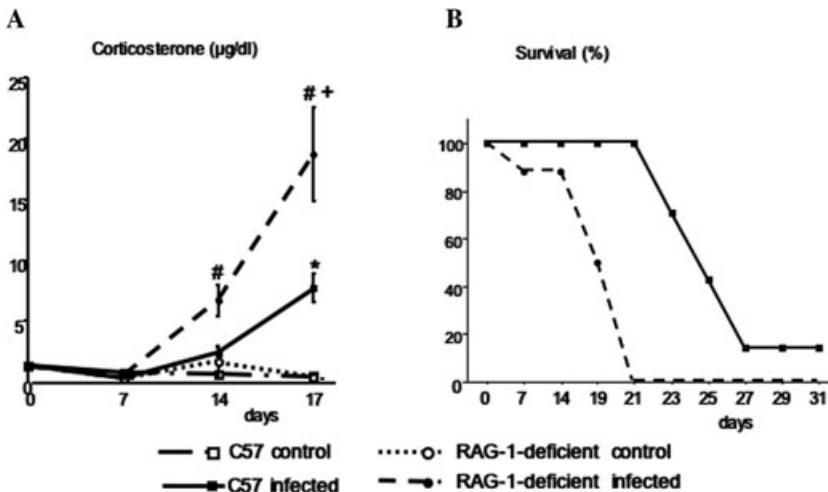
elevated in the immunodeficient mice on day 14 p.i., compared with noninfected controls. All infected RAG-1-deficient mice were dead by day 21 after infection, whereas all infected C57 mice were still alive at this time (Fig. 2B).

NA, 5-HT, and 5-HIAA levels were evaluated on day 18 p.i. in the spleen, inguinal lymph nodes, and heart. NA concentration in the spleen of noninfected RAG-1-deficient mice was significantly higher than in C57 mice (Fig. 3). This difference was no longer significant when values were expressed as total splenic NA content (data not shown), probably due to a nonsignificant tendency of the spleen of RAG-1-deficient mice to be smaller than in normal mice. This way of expressing data of neurotransmitters in the spleen is, however, questionable considering that this organ can accumulate plasma and erythrocytes during the increase in blood flow that parallels immune cell activation.<sup>7</sup> Nevertheless, a statistically significant reduction in splenic NA expressed both as concentration and as total content was observed in both types of mice after infection (Fig. 3). NA concentration in lymph nodes was increased in infected RAG-1-deficient mice when compared with that of noninfected controls and with infected C57 mice. Although NA concentration was decreased in

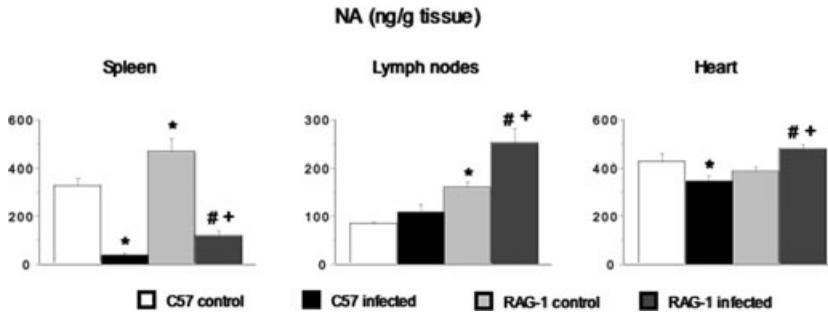
the heart of *T. cruzi*-infected C57 mice, it was increased in infected RAG-1-deficient mice (Fig. 3).

Serotonin concentration was lower in the spleen of noninfected RAG-1-deficient mice compared with C57 mice (Fig. 4). Following infection, the levels were reduced in C57 but increased in immune-deficient mice. In addition, the basal concentration of the serotonin metabolite 5-HIAA was higher in RAG-1-deficient mice than in C57 as well as the ratio HIAA/5-HT (not shown), indicating an increased serotonin turnover rate. However, there was no difference in this ratio between these two strains after infection. Basal concentrations of tryptophan, the serotonin precursor, were higher in RAG-1-deficient than in C57 mice. After infection, tryptophan concentration increased in both types of mice, but was lower in the immune-deficient than in C57 mice (data not shown).

Basal 5-HT concentration in lymph nodes of RAG-1-deficient mice was about sixfold higher than in C57 mice, and infection resulted in a significant reduction (Fig. 4). No significant differences due to the infection were observed in normal mice, but concentration of 5-HIAA was reduced in both strains of mice. Because changes in both 5-HT and its metabolite were proportional in infected and



**Figure 2.** Corticosterone blood levels and mortality of infected C57Bl/6J and RAG-1-deficient mice. (A) Corticosterone levels in plasma were determined in C57Bl/6J and RAG-1-deficient mice (day 0); immediately after, mice were inoculated with vehicle (control) or 100 trypomastigotes (infected). Corticosterone levels in the same mice were also evaluated 7, 14, and 17 days after inoculation. Results are expressed as mean ± SEM. (B) C57 (*n* = 7) and RAG-1-deficient (*n* = 8) were infected with 100 trypomastigotes and left undisturbed. The percentage of mice living at each given time is indicated in the curves. \* *P* < 0.05 versus C57 control; # *P* < 0.05 versus RAG-1-deficient control; + *P* < 0.05 versus C57 infected.



**Figure 3.** NA concentration in the spleen, inguinal lymph nodes, and heart of C57Bl/6J and RAG-1-deficient mice. C57Bl/6J (C57) and RAG-1-deficient (RAG-1) mice were sacrificed 18 days after injection of 100 trypomastigotes (infected) or the vehicle alone (control). NA concentration was evaluated in the spleen, lymph nodes, and heart. Results are expressed as mean  $\pm$  SEM of determinations performed in 6–7 mice per group. \* $P < 0.05$  versus C57 control; # $P < 0.05$  versus RAG-1-deficient control; + $P < 0.05$  versus C57 infected.

noninfected mice of both types, the 5-HIAA/5-HT ratio was not changed, indicating a balanced production and utilization of serotonin. Tryptophan concentration was lower in the lymph nodes of RAG-1-deficient than in C57 mice, but significantly increased after the infection (data not shown).

Basal serotonin concentration in the heart was lower in RAG-1-deficient than in C57 mice. Infection resulted in decreased 5-HT concentrations in both types of animals, but it was significantly lower in the immune deficient than in the normal mice. A comparable pattern was observed for 5-HIAA concentrations (Fig. 4).

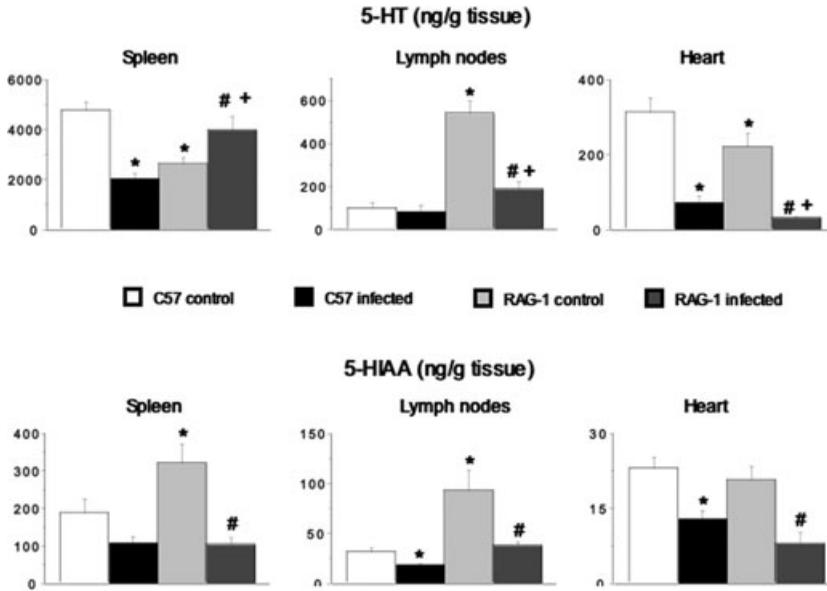
## Discussion

RAG-1-deficient mice have been used as a model to explore natural immunity in the absence of adaptive immunity.<sup>8</sup> In the studies reported here, we use this model to explore possible differences in the response of the HPA axis and the levels of NA and 5-HT following infection with *T. cruzi*. The higher parasitemia and earlier mortality of RAG-1-deficient mice inoculated with *T. cruzi* as compared with the wild-type mice attest for the relevance of adaptive immunity in the control of the infection. Such control is mainly achieved by Th1, Th17, and CD8<sup>+</sup> T cells that recognize and respond to *T. cruzi*-specific antigens.<sup>4</sup> These defense mechanisms cannot operate in RAG-1-deficient mice because they lack mature B and T lymphocytes. Thus, immune defenses in RAG-1-deficient mice are mainly restricted to innate immunity. This ancient defense mechanism is predominantly activated by a MyD88-dependent mechanism, but there are indications that also TRIF-

dependent innate activation pathways contribute to control *T. cruzi* infection.<sup>4</sup> However, activation of the toll-like receptors TLR2 and TLR9 by PAMP products of *T. cruzi* play a predominant role in host defenses against the parasite. The cross-talk between innate and adaptive immunity is also interrupted in RAG-1-deficient mice.<sup>4</sup>

There is now conclusive evidence that immune responses to infectious agents, for example, during pulmonary tuberculosis,<sup>9</sup> are paralleled by complex neuroendocrine host responses that can affect the operation of immune cells. There are also indications that these neuroendocrine responses are, to a large extent, caused by the immune response itself rather than by the infective agent because they can also be elicited by innocuous, noninfective antigens.<sup>10</sup> Furthermore, several cytokines can affect the functioning of the HPA and other endocrine axes, and of the autonomic nervous system (for review, see Ref. 11). We have previously shown that the HPA axis is strongly activated in normal mice during *T. cruzi* infection.<sup>1</sup> Although the increase in endogenous glucocorticoids induces thymus atrophy, it is to a certain extent beneficial for the host because its interference results in an uncontrolled production of proinflammatory cytokines and in earlier death. We have also detected alterations in the SNS during *T. cruzi* infection of immune competent hosts and found that they are also relevant for the course of the disease (manuscript submitted).

The body weight was comparable in noninfected RAG-1-deficient mice and wild-type C57 mice, indicating that, under protected laboratory conditions, their immunodeficiency is partially



**Figure 4.** Serotonin and 5-HIAA concentration in the spleen, inguinal lymph nodes, and heart of C57Bl/6J and (RAG-1)-deficient mice. The concentration of serotonin (5-HT) and its main metabolite 5-HIAA was determined in the spleen, lymph nodes, and heart of the same animals from which the results shown in Figure 3 were obtained. Results are expressed as mean  $\pm$  SEM of determinations performed in 6–7 mice per group. \* $P < 0.05$  versus C57 control; # $P < 0.05$  versus RAG-1-deficient control; + $P < 0.05$  versus C57 infected.

compensated. Infected RAG-1-deficient mice had several fold higher parasitemia, and IL-1 and IL-6 blood levels, than infected normal mice. Because it has been reported that serotonin stimulates the production of these cytokines,<sup>12</sup> our results showing that splenic serotonergic activity is enhanced in immune-deficient mice would agree with this finding. However, the same group reported that serotonin inhibits TNF- $\alpha$  production. In our studies, we found that, although also very elevated, TNF- $\alpha$  levels are comparable in both types of mice after infection.

The response of the HPA axis to *T. cruzi* infection occurred earlier and was stronger in RAG-1-deficient than in C57 mice. This effect is most likely caused by exacerbated IL-1 $\beta$  and IL-6 production in infected RAG-1-deficient mice, and indicates that the HPA axis, which, as we have shown operates in normal mice infected with *T. cruzi*,<sup>1</sup> is even overactivated in animals that have no functional T and B lymphocytes.

Basal NA concentrations were higher in the spleen of noninfected RAG-1-deficient than in C57 mice. However, the splenic NA concentration and content were decreased in both after infection. Such de-

crease during prolonged enhanced immune activity is, in general, a reflection of the loss of noradrenergic nerve fibers, as observed, for example, in a model of lymphoproliferative disease,<sup>13</sup> and in target organs of inflammatory process such as arthritic joints.<sup>14</sup> Such loss has also been observed in the spleen of normal mice infected with *T. cruzi* (manuscript submitted). The results reported here indicate that cells other than mature T or B cells, or their products, contribute to this effect.

Even though the NA content in lymph nodes was comparable because the immunodeficient animals have smaller lymph nodes, the concentration of the neurotransmitter was increased in RAG-1-deficient mice, compared with normal mice. This difference is even more marked when animals are infected. This pattern indicates that resident cells, which are not mature T and B lymphocytes, in the lymph nodes of RAG-1-deficient mice are exposed to higher concentrations of NA than in normal mice.

The basal serotonin concentration in the lymph nodes was about fivefold higher in RAG-1-deficient than in normal animals. As in the spleen, the lymph nodes of these immune-deficient animals have also higher basal levels of its metabolite 5-HIAA,

indicating that the absence of mature T and B lymphocytes is related to such difference. Following infection, serotonin levels increased in the spleen of the immune deficient mice but decreased in the lymph nodes. However, these low values were still higher when compared with those of infected normal mice. Decreased 5-HIAA concentrations in the spleen and lymph nodes were detected in both types of mice after infection. However, the ratio of 5-HIAA/5-HT only significantly decreased in the spleen of the infected RAG-1-deficient mice, probably indicating reduced utilization of indolamine. Serotonergic nerves were found in the enteric nerve system,<sup>15</sup> pancreas,<sup>16</sup> and iris.<sup>17</sup> However, so far there is no evidence that serotonergic nerve fibres are present in the spleen and lymph nodes, although there are indications that serotonin can be costored with NA and released following sympathetic nerve stimulation.<sup>18</sup> Thus, the most likely source of serotonin in these organs is platelets, which store 5-HT synthesized by enterochromaffin cells in the gut<sup>19</sup> and mast cells.<sup>20</sup> It has been shown that activated T lymphocytes can synthesize serotonin.<sup>21</sup> However, the results reported here indicate that T lymphocyte-derived serotonin seems not to be a main source of this indolamine. Indeed, under basal conditions, RAG-1-deficient mice had even higher concentration of serotonin in the lymph nodes than normal mice, and its concentration was elevated in the spleen even after infection with *T. cruzi*.

Cardiomegaly, a feature of Chagas disease, is attributed to local inflammatory processes linked to TLR2-induced NF- $\kappa$ B activation and local IL-1 production.<sup>6</sup> The increased weight of the heart relative to the body weight observed in C57 mice 17 days after *T. cruzi* inoculation seems to reproduce this situation in the experimental model used. Conversely, a significant decrease in the absolute and relative weight of the heart of RAG-1-deficient mice was observed despite the fact that these animals have more parasites in their blood. Interestingly, it has been reported that anti- $\beta$ -1 adrenergic receptor antibodies seem to contribute to the pathogenesis of dilated cardiomyopathy and heart failure in Chagas disease.<sup>22–24</sup> These antibodies, which could interfere with anti-inflammatory effects of catecholamines, cannot be produced by RAG-1-deficient mice. The absence of cardiomegaly

in RAG-1 mice might therefore be explained by their incapacity to produce anti- $\beta$ -1 adrenergic receptor antibodies. However, it remains to be explained why, instead of cardiomegaly, the weight of the heart is reduced in *T. cruzi*-infected immune-deficient mice. Such an effect might be related to the much higher production of proinflammatory cytokines in these mice. In fact, it has been shown that IL-1 and TNF- $\alpha$  in particular can induce cardiomyocyte death.<sup>25–29</sup> Furthermore, RAG-1-deficient mice do not produce enough anti-inflammatory cytokines, such as IL-10, that could counteract the proapoptotic effects of IL-1 and TNF- $\alpha$  on cardiac cells.<sup>30,31</sup>

In conclusion, our result suggests that the absence of adaptive immunity, as is the case in RAG-1-deficient mice, results in alterations in some neuroendocrine parameters under basal conditions and in response to *T. cruzi* infection. In our view, this is conceptually relevant not only because it provides further indications that neuroendocrine responses during infections are mediated by immune-derived products, but also because it stresses that the final outcome of these responses is based on interactions between natural and adaptive immunity. Indeed, the role of natural immunity following infection seems to be exacerbated in RAG-1-deficient mice, as reflected by higher levels of IL-1 $\beta$  and IL-6 than in normal mice. As discussed previously, although this was not the case for TNF- $\alpha$ , such increase might be explained by the inhibitory effect of serotonin, which have mixed effects on cytokine production and on natural immunity in general.<sup>18,32</sup> The endogenous production of glucocorticoids is increased earlier and the levels are higher in RAG-1-deficient than in C57 mice, and the results suggest that there are also differences in the activity of the SNS in lymphoid organs, both under basal conditions and following *T. cruzi* infection. At present, we are studying the significance of the different pattern of alterations in the levels of NA and 5-HT in the spleen, lymph nodes, and heart between RAG-1-deficient and C57 mice for the course of the disease. However, the high levels of corticosterone in blood attained in infected RAG-1-deficient mice already indicate that this anti-inflammatory hormone mediates, although not enough, an exacerbated host response to restrict the inflammatory component of the disease caused by the parasite.

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## Conflicts of interest

The authors declare no conflicts of interest.

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