

Previous contact with *Strongyloides venezuelensis* contributed to prevent insulinitis in MLD-STZ diabetes

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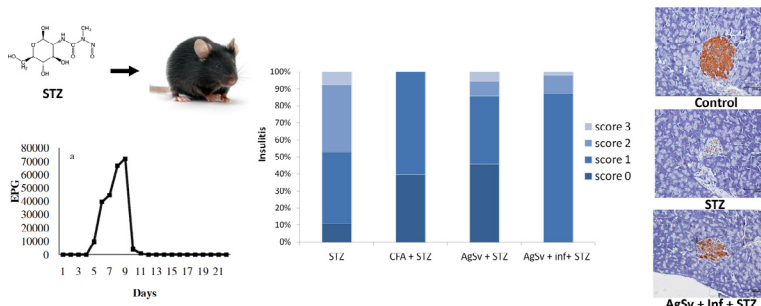
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HIGHLIGHTS

- Recovery from *S. venezuelensis* infection is associated with Th2 polarized response.
- Contact with *S. venezuelensis* contributed to prevent insulinitis in MLD-STZ diabetes.
- Protection associated with *S. venezuelensis* was linked to IL-5 and IL-10 production.

GRAPHICAL ABSTRACT



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ABSTRACT

Epidemiological and experimental studies support the idea that helminth infections can induce a protective effect against the development of autoimmune and allergic diseases. In this study we characterized the immune response induced by *Strongyloides venezuelensis* infection in C57BL/6 mice and then evaluated the effect of a previous contact with this helminth in the outcome of type 1 diabetes. Animals were initially infected with 2000 L3 larvae from *S. venezuelensis* and euthanized 22 days later. An acute phase, identified by a high amount of eggs per gram of feces, was established between days 7 and 9 post-infection. Recovery from infection was associated with a Th2 polarized response characterized by a significant level of serum IgG1 specific antibodies and also a significant production of IL-5 and IL-10 by spleen cells stimulated with *S. venezuelensis* soluble antigen. Immunization with soluble *S. venezuelensis* antigen associated with complete Freund's adjuvant followed by infection with *S. venezuelensis* protected mice from diabetes development induced by streptozotocin. Protection was characterized by a higher body weight gain, lower glycemic levels, much less severe insulinitis and preserved insulin production. Together, these results indicate that *S. venezuelensis* contributed to protect C57BL/6 mice against experimental diabetes induced by streptozotocin.

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1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder associated, in genetically susceptible individuals, with generation and activation of autoreactive T cells that recognize pancreatic β -cell autoantigens. Self-reactive CD4⁺ and CD8⁺ T lymphocytes infiltrate

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the pancreas and selectively destroy the insulin producing β -cells in the islets (Chentoufi et al., 2008). T1D has clearly increased in prevalence over the last several years in developed countries (Shapira et al., 2010). Mice and rats have been widely used as experimental models to investigate the contribution of helminths and other environmental agents to regulate the immune system during allergy and autoimmune diseases. Epidemiological evidences strongly suggest that improved standards of living are associated with an increased incidence of immune system-mediated diseases as allergies and autoimmune pathologies. A theory known as the “hygiene hypothesis” suggests that improved health standards through sanitation and vaccination may in part be responsible for the apparent increase in immune-mediated diseases due to decreased microbiological and parasitic infections in humans, particularly in children (Whary and Fox, 2004). Thus, parasitic infections might somehow shape the immune system to avoid exaggerated inflammatory responses (Cooke et al., 2004; Romani, 2008; Vercelli, 2006). Epidemiological and experimental studies have supported the idea that helminth infections can induce a protective effect against the development of both autoimmune and allergic diseases (Zaccone et al., 2008).

Species of *Strongyloides* are important intestinal parasites of humans and domestic animals (Grove, 1996; Júnior et al., 2006). *S. venezuelensis* is a rodent parasite, usually found in rats and is very useful as a model to study nematode infections (Marra et al., 2010; Maruyama et al., 2006). Infective larvae of *S. venezuelensis* penetrate into the skin and migrate to the lungs where they achieve the fourth stage. Then, these larvae reach the small intestine where they finally become adult parasites (Tindall and Wilson, 1988). Our previous experience with this parasite indicated that it was able, as many other nematodes, to induce a strong Th2 kind of response in Lewis rats (Chiuso-Minicucci et al., 2010). Streptozotocin (STZ) is a nitrosourea antineoplastic agent that exhibits direct pancreatic β -cell cytotoxicity (Rerup, 1970). This substance has been widely used to induce two distinct experimental forms of diabetes. An inflammatory form of diabetes with clinical and immunohistological features similar to those found in human type 1 diabetes, can be induced by injection of multiple low doses of STZ in susceptible strains of mice (Kolb, 1987; Like and Rossini, 1976).

The purpose of this study was to determine if *S. venezuelensis* infection was able to induce a strong Th2 response in C57BL/6 mice as we observed in Lewis rats. In addition, as accentuated Th2 profiles have been associated with immunomodulatory ability, we also tested the effect of this infection on the outcome of experimental autoimmune diabetes.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were purchased from CEMIB (UNICAMP, Campinas, SP, Brazil). Mice received sterilized food and water *ad libitum* and were manipulated in compliance with the ethical guidelines adopted by the Brazilian College of Animal Experimentation, being the experimental protocol approved by the local Ethics Committee (protocol 84/08).

2.2. General experimental protocol

Mice were initially infected with *S. venezuelensis* to determine the kinetics of the infection. They were euthanized 22 days later to characterize the immune response. The effect of a previous contact with this helminth on the development of experimental diabetes was then evaluated. This contact was established by immunization with soluble *S. venezuelensis* antigens followed by infection with this worm. Diabetes was induced seven days later

by giving STZ during 5 consecutive days by intraperitoneal route. Twenty-one days after last STZ injection, animals were euthanized and diabetes intensity was quantified by increase in body weight, glycemic levels, insulinitis score and *in situ* insulin production.

2.3. Immunization and infection with *S. venezuelensis*

The *S. venezuelensis* strain was isolated from wild rats in 1980. The strain was then maintained in Wistar rats, routinely infected in the Parasitology Laboratory of the Univ. Estadual Paulista (UNESP). For the experimental infections, infective third-stage larvae (L3) of *S. venezuelensis* were obtained from fecal cultures using sterilized horse manure as substrate. The cultures were incubated at 25 °C for 72 h and the infective larvae were collected and concentrated by using a Baermann apparatus. Recovered larvae were washed in phosphate-buffered saline (PBS) and their number was estimated under stereo-microscopy. These L3 were used for both, soluble antigen preparation and infection. To obtain soluble antigen as previously described (Fernandes et al., 2008), larvae were resuspended in RPMI medium containing a protease inhibitor cocktail (Roche Applied Science, Mannheim, Baden-Wurtemberg, Germany) and disrupted by vortexing with glass beads (five cycles of 1 min each) followed by sonication (10 cycles of 1 min) with a cell sonic disrupter (Vibra Cell Ultrasonic Processor VCX-400). After removing the insoluble particles by centrifuging the larvae homogenate, the recovered supernatant was filtered through a 0.22 μ m membrane (Millipore) and aliquots were stored at –80 °C. The protein concentration was determined by bicinchoninic acid assay (Bicinchoninic Acid Kit for protein determination-Sigma, St. Louis, MO, USA). This antigen was used to estimate antibody levels, to stimulate spleen cell cultures *in vitro* and also to immunize the animals. Mice were immunized with 50 μ g of L3 antigen in complete Freund's adjuvant (CFA) and one week later they were subcutaneously infected at the abdominal region with 2000 L3 larvae.

2.4. Fecal egg count

Infection intensity was determined by counting the number of eggs per gram of feces (EPG) by a modified Cornell McMaster method (Gordon and Whitlock, 1939). Fecal samples were daily collected until 22 days after infection. Infected animals were allocated in a box and their feces were collected three hours later to determine the number of eggs.

2.5. Parasite-specific antibodies

Serum parasite-specific IgG1 and IgG2a were estimated by ELISA. Briefly, plates (Nunc, Life Tech. Inc., USA) were coated with 100 μ g/mL of L3 antigen in coating solution ($\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.6), at 4 °C, overnight. Non-specific protein binding was blocked by incubation with 200 μ l of 0.05% tween 20, 10% fetal calf serum (FCS) in PBS for 1 h at 37 °C. Subsequently, plates were incubated with serum diluted 1:10 during 1 h at 37 °C. For the detection of specific IgG1 and IgG2a subclasses, the plates were incubated with biotinylated rat antimouse antibodies specific for each isotype (PharMingen, BD Biosciences, USA) for 1 h at 37 °C. Plates were then incubated for 30 min at room temperature with Strept AB (kit from Dako, Carpinteria), and revealed by adding H_2O_2 and o-phenylenediamine (Sigma, USA). Color development was stopped with H_2SO_4 and optical density was measured at 492 nm.

2.6. Cytokine production by spleen cells

Spleen cells were collected and adjusted to 5×10^6 cells/mL. They were then cultured in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine and 40 mg/L of gentamicin, in the

presence of 100 µg/mL of *S. venezuelensis* L3 soluble antigen or 10 µg/mL of concanavalin A (ConA, Sigma, St. Louis, MO, USA). Cytokine levels were evaluated 72 h later by ELISA, in culture supernatants, according to manufacturer instructions (BD Biosciences, San Jose, California, USA). Sensitivity of ELISA for IFN-γ, IL-5 and IL-10 were 16; 8 and 16 pg/mL, respectively.

2.7. Analysis of weight gain and glycemic levels in STZ-induced diabetes

In order to induce diabetes, male C57BL/6 mice were given daily intraperitoneal injections of STZ diluted in citrate buffer, (40 mg/kg, Sigma–Aldrich, St. Louis, MO) for five consecutive days. Body weight was evaluated twice a week. Non-fasted serum glucose concentration was measured using Prestige LX Smart System Test-strips (Home Diagnostic, Inc., Fort Lauderdale, FL) once a week, beginning 7 days after the last STZ dose. The animals were considered diabetic when glucose blood level was higher than 200 mg/dL during 2 consecutive weeks.

2.8. Histopathological analysis

Three weeks after the last STZ injection, the pancreata were removed and fixed in buffered formalin 10% (phosphate buffer pH = 7.2) for 24–48 h. The organs were conserved in alcohol 70% until histological processing and paraffin inclusion. Five micrometer sections were cut and stained with hematoxylin and eosin (HE). Inflammatory infiltrates were quantitatively evaluated using a computer-assisted image system based on a Nikon Microphot-FXA optical microscope connected via a Sony Exwave HAD video camera to a computer. Total section area of each pancreas was measured to avoid any inter-animal variance. Islets and inflammation areas were measured individually (Kauri et al., 2007) using the KS300 software (Carl-Zeiss, AG, Germany). The islet's area showing mononuclear cell infiltrate was then converted into relative values (%) according to the formula: mononuclear infiltrate area (µm²) × 100/islet's area (µm²). The results were further categorized into a specific score: 0 = intact islet; 1 = peri-insulinitis; 2 = moderate insulinitis (<50% of the islet infiltrated) and 3 = severe

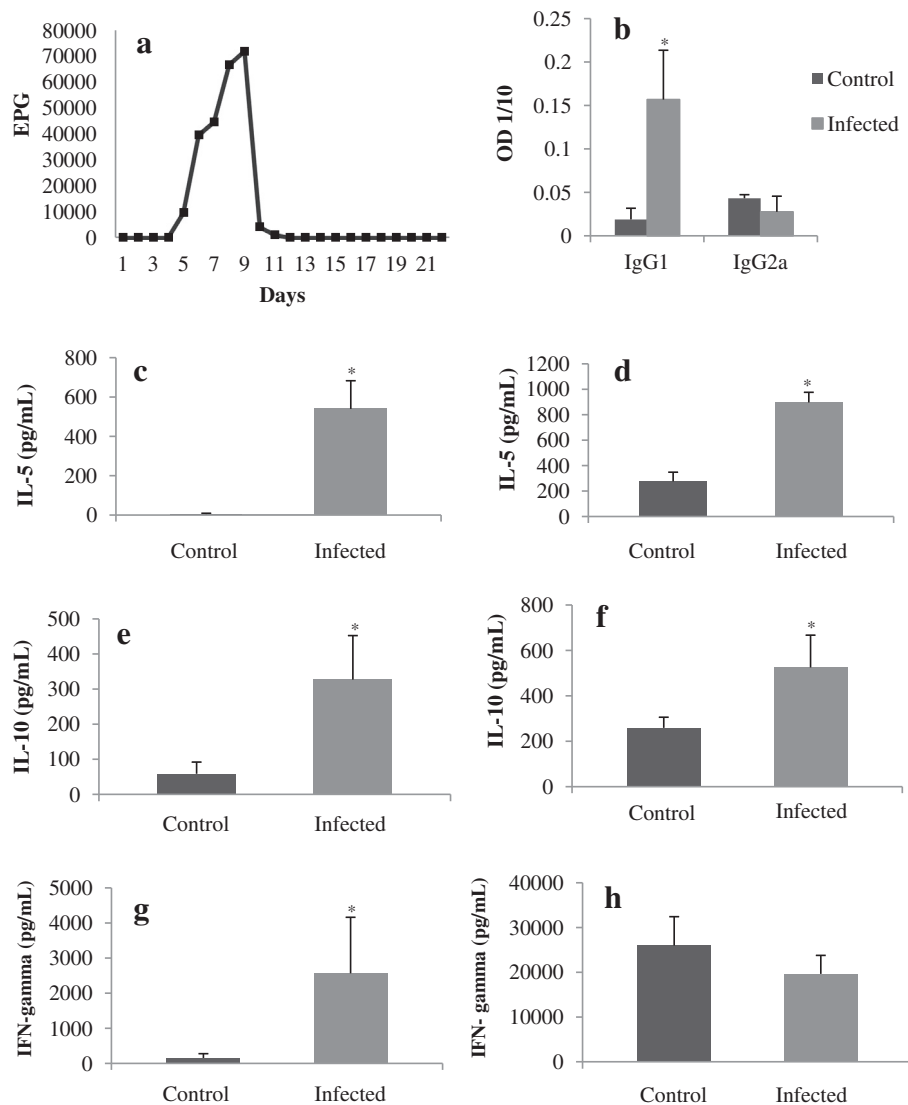


Fig. 1. Characterization of the immune response during the recovery phase of *S. venezuelensis* infection. The number of eggs per gram of feces was daily determined in a fecal pool (a). Specific serum IgG1 and IgG2a (b), specific cytokine induction: IL-5 (c); IL-10 (e); IFN-γ (g) and ConA cytokine induction: IL-5 (d); IL-10 (f) and IFN-γ (h) were evaluated by ELISA in spleen culture supernatants. Data represents the mean ± SD from 6 animals. **p* < 0.05 in comparison to control group.

insulinitis ($\geq 50\%$ of the islet infiltrated). At least 10 islets per pancreas were analyzed in non-consecutive sections.

2.9. Insulin detection

To evaluate the islet's insulin content, pancreas slides were initially treated with 0.01 M citrate buffer (pH 6.0) and heated twice, 5 min each time, in a microwave oven. Slides were then incubated with 3% H₂O₂ in PBS for 10 min and non-fat milk for 60 min, to block endogenous peroxidase and endogenous biotin, respectively. Insulin content was revealed by incubation with

polyclonal guinea pig anti-insulin antibody (dilution 1:500, Dako Glostrup, Denmark) overnight and then with rabbit anti-guinea pig IgG coupled with peroxidase (Dako, Glostrup, Denmark). Chromogen color development was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The slides were counterstained with Harris's hematoxylin and morphometric analysis was performed with a computer-assisted image system, as described above. The islet's insulin content was established by calculating the area of immunopositive insulin relative to the islet's area, according to the formula: insulin positive area (μm^2) \times 100/islet's area (μm^2).

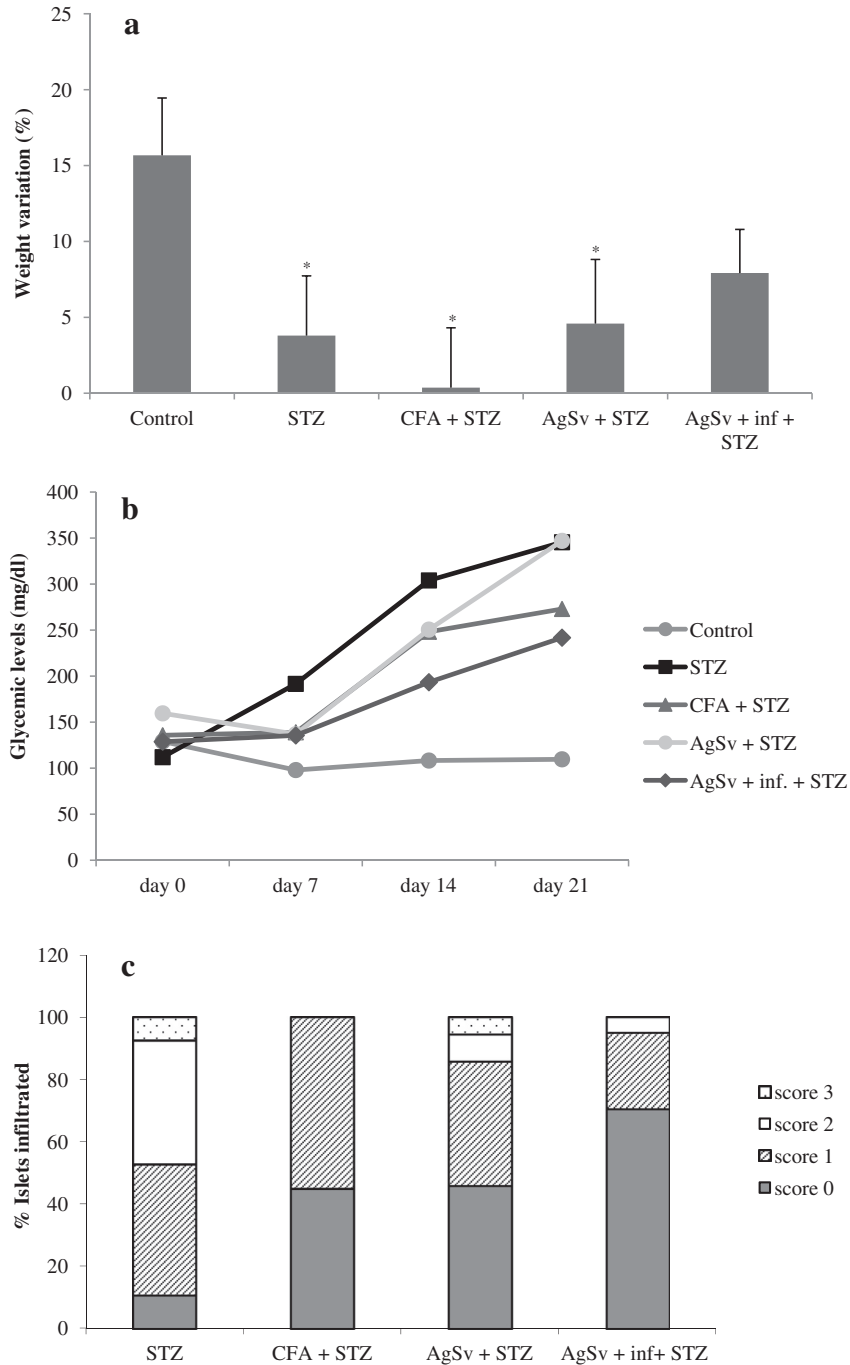


Fig. 2. Clinical and histopathological alterations triggered by STZ in C57BL/6 mice previously immunized and infected with *S. venezuelensis*. Body weight was measured twice a week and the percentage of weight variation was determined based on day 0 and day 21 (a) and glycemic levels were checked once a week (b). Insulinitis score was determined three weeks after last STZ dose (c). Results represent the average among 6 animals. * $p < 0,05$ in comparison to the control group.

2.10. Statistical analysis

Data were expressed as mean ± SD. Comparisons between groups were made by Student's *t* test or one way ANOVA with post hoc Holm-Sidak test for parameters with normal distribution, and by Mann-Whitney *U* test or Kruskal-Wallis test for parameters with non-normal distribution. Significance level was $p < 0.05$. Statistical analysis was accomplished with SigmaStat for Windows v 3.5 (Systat Software Inc).

3. Results

3.1. Recovery from *S. venezuelensis* infection is associated with a Th2 polarized response

As can be observed in Fig. 1a, C57BL/6 mice presented an acute infection characterized by a high amount of EPG between days 7 and 9 post infection. From the 12th day on, no more eggs were

detected. Even though antibody levels were low, there was a significant production of IgG1, but not IgG2a, during the recovery phase (Fig. 1b). IL-5, IL-10 and IFN- γ production induced by specific antigen stimulation (Fig. 1c, e and g, respectively) were significantly elevated during this phase. A significant increase was also observed in the production of IL-5 and IL-10 (Fig. 1d and f, respectively) by spleen cell cultures stimulated with ConA. IFN- γ levels induced by ConA were similar in the control and infected groups (Fig. 1h).

3.2. *S. venezuelensis* contributed to protect against diabetes

Immunization of C57BL/6 mice with soluble antigens from *S. venezuelensis* associated with CFA, followed one week later by infection with 2000 L3 from this worm, determined a clear protection from diabetes development induced by STZ. As expected, STZ inoculation clearly avoided normal body weight increase, i.e., whereas the control group gained 15.7% of their original weight,

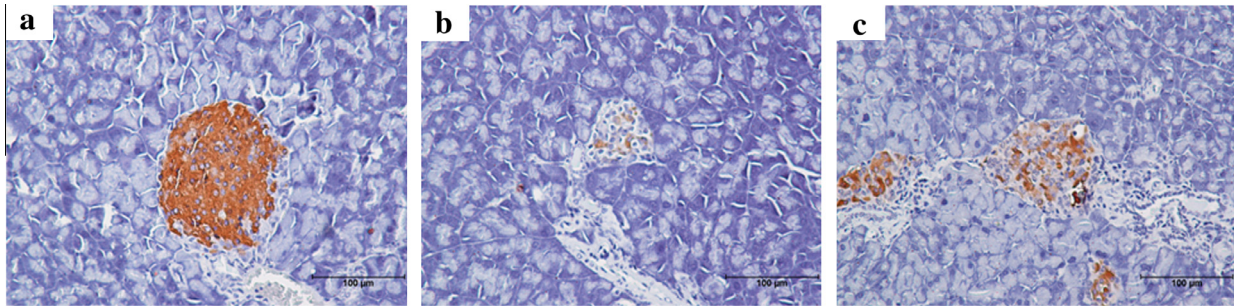


Fig. 3. *In situ* production of insulin in mice protected against diabetes by contact with *S. venezuelensis*. Animals were immunized with *Strongyloides* soluble antigen associated with CFA and then infected with *S. venezuelensis*. Three weeks after infection, diabetes was induced by STZ, the pancreata were collected and insulin production detected by immunohistochemistry. Normal control (a), STZ (b) and AgSv+inf+STZ (c).

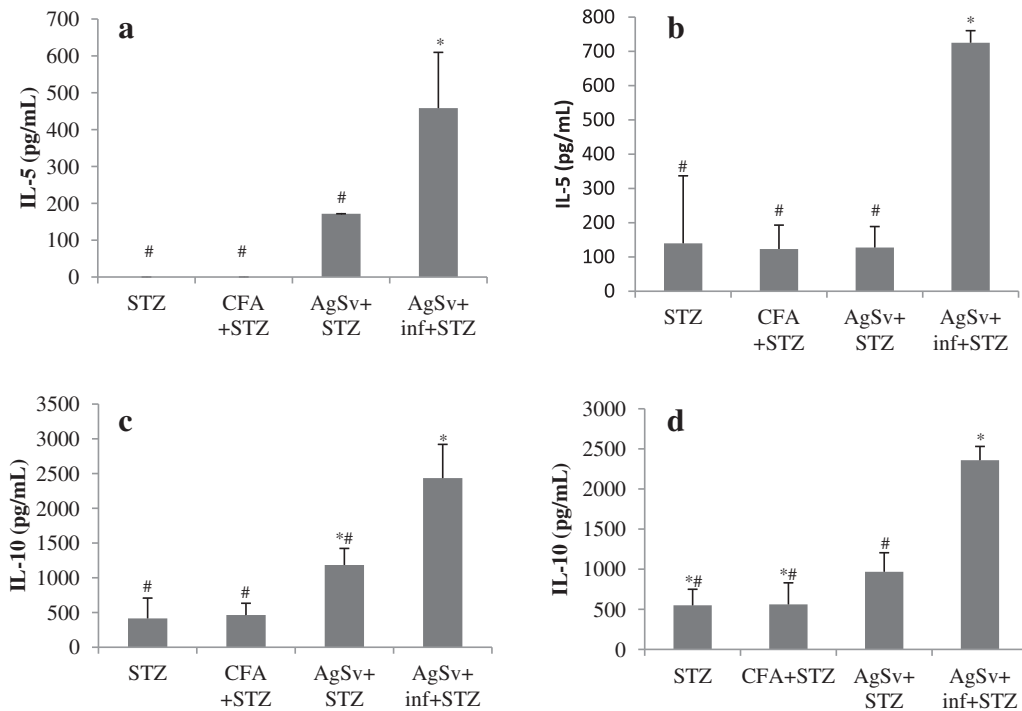


Fig. 4. Production of cytokines in mice protected against diabetes by contact with *S. venezuelensis*. Cytokine production induced by soluble *S. venezuelensis* antigen: IL-5 (a); IL-10 (c) and cytokine production induced by ConA: IL-5 (b); IL-10 (d) were evaluated in spleen cell cultures. Data represent the mean ± SD from 6 animals. Different symbols indicate significant difference between groups. * $p < 0.05$.

STZ group gained only 3.8%. Groups injected with CFA+STZ or AgSv+STZ also gained significantly less weight during the experimental period, 0.4% and 4.6%, respectively. On the other hand, the group that was immunized and later infected with *S. venezuelensis* before STZ inoculation significantly preserved this weight gain (Fig. 2a).

The clearest differences in glycemic levels were observed 14 days after STZ inoculation. As showed in Fig. 2b, among the STZ groups, only the one previously immunized and then infected with *S. venezuelensis* (AgSv+inf+STZ) presented glycemic levels below 200 mg/dL. This value was statistically different from the value found in the STZ group. Even though a similar profile was observed at the 21st day after diabetes induction, no statistical difference was detected among the groups.

The three procedures protected against insulinitis. However, immunization followed by infection with *S. venezuelensis* determined the most significant effect. In this case, 70% of the islets remained at score zero, i.e., they presented no inflammation. In mice injected with CFA associated or not with *S. venezuelensis* antigens the percentage of islets with score zero was around 40%. Otherwise, only 10% of the islets remained preserved in the STZ control group (Fig. 2c).

3.3. Insulin detection

As expected and showed in Fig. 3a, a widespread immunohistochemical detection of insulin was observed in normal pancreas. In contrast, staining for insulin was very discrete in pancreas obtained from mice submitted to diabetes development by STZ inoculation (Fig. 3b). Previous contact with CFA alone (CFA+STZ) or associated with *S. venezuelensis* antigens (AgSv+STZ and AgSv+inf+STZ groups) highly preserved the ability to produce insulin. Even though insulin staining seemed more preserved in the AgSv+inf+STZ group, a quantitative analysis indicated no statistical difference in comparison to the CFA+STZ group. Staining for insulin in AgSv+inf+STZ group is illustrated in Fig. 3c.

3.4. Protection associated with *S. venezuelensis* was linked to higher IL-5 and IL-10 production

Production of IL-5 (Fig. 4a) and IL-10 (Fig. 4c) induced by *in vitro* stimulation of spleen cells with soluble *S. venezuelensis* antigen was higher in the two groups that had a previous contact with worm antigens by immunization followed or not by infection. Comparison between AgSv+STZ and AgSv+inf+STZ groups indicated that IL-5, but not IL-10 levels, were statistically higher in AgSv+inf+STZ group. In spleen cell cultures polyclonally stimulated with ConA, there was a significant production of both cytokines only in the AgSv+inf+STZ group. IL-5 and IL-10 production can be observed in Fig. 4b and d, respectively.

4. Discussion

Helminths are a highly diverse group of organisms with distinct morphologies and life cycle stages that are able to cause a multiplicity of diseases in humans and animals (Schiefke et al., 2006). Despite many differences, they share the potential to trigger profound regulatory effects over the immune system (Cooke et al., 1999; Flohr et al., 2009). In this investigation we evaluated the ability of *S. venezuelensis* to modulate the development of experimental diabetes induced by multiple streptozotocin (STZ) doses in C57BL/6 mice. Male C57BL/6 mice were initially infected with 2000 L3 from *S. venezuelensis* by subcutaneous route being the kinetics of the infection determined by counting the number of eggs per gram of feces (EPG). This analysis indicated the presence

of an acute phase between days 7 and 9 after injection, followed by a recovery phase whose immunological status was characterized 22 days after infection. Even though there was an specific induction of IFN- γ during this phase, elevated specific induction of IL-5 and IL-10 and also significant IgG1 production characterized a predominant Th2 profile during this period. This profile was also reinforced by elevated production of IL-5 and IL-10, but not IFN- γ , after ConA stimulation. These findings demonstrated that strongyloidiasis in C57BL/6 mice is similar to other rodent helminthiasis (Baek et al., 2003; Chiuseo-Minicucci et al., 2010; Kimura et al., 1999) and also that it establishes a Th2 environment as described for other worms (Chiuseo-Minicucci et al., 2010; Machado et al., 2005; Machado et al., 2007).

To evaluate the effect of a previous contact with *S. venezuelensis* on diabetes, mice were immunized or infected with this worm before STZ disease induction. Previous infection by itself induced a very discrete protective effect (not shown). However, the stronger stimulation achieved by immunization with soluble *S. venezuelensis* antigens followed by infection with this worm, triggered a significant protective effect. In this case, the animals gained more weight, presented glucose levels below the ones found in STZ control group and also displayed a striking preservation of the pancreatic islets. Seventy percent of the islets were completely preserved in previously immunized/infected groups whereas the STZ control group presented only 10% of islets with zero score. In addition to this structural preservation, this experimental group also presented the highest percentage of islets area producing insulin. Comparing to the control group, whose total insulin producing area was considered 100%, the percentages of areas able to produce this hormone were 14%, 66.7% and 74.3% in STZ, CFA+STZ and AgSv+inf+STZ groups, respectively. Similar protective findings mediated by other helminths were also reported in this model. For example, El-Wakil et al., 2002, demonstrated that *Schistosoma mansoni* egg deposition, which leads to a shift from a Th1 to a Th2 profile, suppressed diabetes development. Also, very recently, Espinoza-Jiménez et al., 2010 demonstrated that *Taenia crassiceps* infection attenuated this disease, determining a lower diabetes incidence, a much smaller degree of insulinitis and also preserved pancreatic insulin production. An expressive level of protection was achieved in our investigation when immunization with soluble *S. venezuelensis* antigens was followed by infection with this helminth. A possible explanation for this requirement could be linked to the fact that *S. venezuelensis* is an acute parasitic disease, usually controlled by the host immune response (Fernandes et al., 2008) whereas *T. crassiceps* and *S. mansoni* determine chronic types of infections (Machado-Silva et al., 2010; Morales-Montor et al., 2001).

It's important to highlight, however, that mice immunized with soluble *S. venezuelensis* antigen emulsified with CFA and their respective control, i.e., mice solely injected with CFA, also present a smaller but significant level of protection. It is possible, therefore, that the high protection triggered by immunization followed by *S. venezuelensis* infection was initiated by the stimulus derived from *S. venezuelensis* soluble antigens or by the CFA itself. Even though mycobacteria and helminths have been both indicated as possible "old friends" of humans beings, able to regulate autoimmunity, their possible association determining a stronger immunoregulatory effect has not being investigated. Further experiments will be necessary to unravel the real contribution of mycobacteria present in CFA to this protective effect.

Theoretically, we could attribute this enhanced protective effect observed in the AgSv+inf+STZ group to the concurrence of distinct mechanisms. On one hand, it is well known that diverse sources of mycobacterial antigens as CFA, BCG and *Mycobacterium avium* are able to prevent experimental diabetes (Brás and Aguas, 1996; Gao et al., 2010; Qin and Singh, 1997; Ryu et al., 2001). A plethora

of mechanisms have been associated with this non-specific immunomodulation, including direct or indirect induction of regulatory cells (Manirarora et al., 2008; McInerney et al., 1991), deviation of Th1 response to non-deleterious Th2 profile (Qin et al., 1998) and up-regulation of TNF- α expression that leads to apoptosis of diabetogenic T cells (Qin et al., 2004). On the other hand, infections with helminths can also be beneficial to the host as they are able to suppress allergic and autoimmune responses (Wilson and Maizels, 2004). Recent reports indicated that the ability of helminths to decrease type 1 diabetes incidence is related to increased activity of Th2 and Treg cells (Hübner et al., 2009; Zaccane et al., 2009).

Quantification of some cytokines in the supernatants of spleen cell cultures stimulated with specific antigen or ConA partially explained the contribution of *S. venezuelensis* to this protection. In cultures stimulated with specific soluble antigen, the AgSv+inf+STZ group produced higher levels of IL-5 and IL-10 in comparison to the group that was only immunized. The same cytokine profile was observed in cultures stimulated with ConA. These findings suggested that both IL-5 and IL-10 could contribute to this protection. Protocols that increase the level of these cytokines have been associated with protection from antiretinal autoimmunity (Cortes et al., 2008), autoimmune diabetes (Perone et al., 2009) and arthritis (van Duivenvoorde et al., 2007).

5. Conclusion

Together these results demonstrate that *S. venezuelensis* was able to increase the ability of CFA to protect against experimental diabetes. These findings suggest that a deeper investigation is mandatory to highlight the meaning of the emerging hypothesis that “old friends” could act in concert to regulate autoimmunity.

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