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Short Communication

Extract of *Ascaris suum* induces TGF-β and early production of IgG1 in experimental autoimmune hepatitis

Extrato de *Ascaris suum* induz TGF-β e produção precoce de IgG1 na hepatite autoimune experimental

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Abstract

In experimental autoimmune hepatitis (EAH) of Th1 profile, an extract of adult *Ascaris suum* worms (ASC) was previously found to deviate the immune response to a Th2/IL-10 pattern. Here, the effects of treatment with ASC on production of TGF-β and the anti-*Ascaris* isotypes IgG1 and IgG2a in EAH were evaluated. EAH was induced in BALB/c mice, intravenously with concanavalin A. Two hours later, these animals received ASC (EAH+ASC group) or PBS vehicle (EAH group). IgG1 and IgG2a were evaluated 8 h, 24 h and 7 d after induction. TGF-β was measured in a splenocyte culture at this last time. The isotype levels in the EAH group were low throughout the kinetics. In the EAH+ASC group, there was significant production of IgG1 at 24 h and 7 d, but of IgG2a only at 7 d. There was statistically greater production of TGF-β in the EAH+ASC group. The higher levels of IgG1 and TGF-β in this group suggest that an additional Th1 response control route exists in EAH, which needs to be investigated.

Keywords: *Ascaris suum*, experimental autoimmune hepatites, immunomodulation.

Resumo

Na hepatite autoimune experimental (HAE) de perfil Th1, o extrato de vermes adultos *Ascaris suum* (ASC) desviou a resposta imune para um padrão Th2/IL-10. Neste trabalho, foram avaliados os efeitos do tratamento com ASC na produção TGF-β e dos isótipos de IgG1 e IgG2a anti-*Ascaris* na HAE. Esta foi induzida em camundongos BALB/c intravenosamente com Concanavalina A. Após duas horas, os animais receberam ASC (grupo HAE+ASC) ou veículo PBS (grupo HAE). IgG1 e IgG2a foram avaliados em 8 horas, 24 horas e 7 dias após indução. TGF-β foi mensurado em cultura de esplenócitos nesse último tempo. Os níveis dos isótipos no grupo HAE foram baixos durante toda a cinética. No grupo HAE+ASC, houve produção significativa de IgG1 em 24 horas e 7 dias, mas somente em 7 dias para IgG2a. A produção de TGF-β foi estatisticamente maior no grupo HAE+ASC. Níveis mais altos de IgG1 e TGF-β nesse grupo sugerem uma via adicional de controle da resposta Th1 na HAE que precisa ser investigada.

Palavras-chave: *Ascaris suum*, hepatite autoimune experimental, imunomodulação.

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Introduction

An extract of adult *Ascaris suum* worms (ASC) has been found to suppress the inflammatory response to heterologous antigens through mechanisms that depend on IL-10/TGF-β and $CD4^+CD25^+Foxp3^+$ T cells (Araújo et al., 2010; Souza et al., 2002). In experimental autoimmune hepatitis (EAH), it has been demonstrated that ASC has a protective effect through promoting increased levels of IL-4, IL-10 and IL-13, accompanied by reduction of inflammatory infiltrate and restoration of transaminases (Nascimento et al., 2014). However, the levels of TGF-β and anti-*Ascaris* IgG isotypes in this inflammation model were not evaluated.

ASC induced Th2 response that was correlated with the *in vivo* suppression, IL-4 and IL-10 synthesis (Ferreira et al., 1995). Immunization with PAS-1, a protein of *A. suum*, stimulated the Th2 response, production of IL-10 and TGF-β in allergic airway inflammation induced by OVA in mice (Araújo et al., 2010). Regarding IgG subclasses, immunization with extract of adult worms, larvae or worm cuticle of *A. suum* confers protective immunity against infection induced by anti-*Ascaris* antibodies of the subclasses IgG1 and IgG3 (Gazzinelli-Guimarães et al., 2018). Immunization with As16, a protein genetically related to *A. suum*, also induces a predominantly Th2 response, with high serological titers of IgG1 (Wei et al., 2017). It is known that IgG isotypes activate macrophages that produce IL-10, thus impairing the Th1 response (Buxbaum & Scott, 2005). It is possible that ASC may help in controlling the autoimmune inflammatory process through inducing a suppressive profile via IgG1/IL-10 and TGF-β. Although it is known that treatment with ASC causes negative modulation of immunopathology in EAH, with development of an immune response of Th2 profile, evaluation of other modulation routes becomes necessary.

Materials and Methods

Animals, *Ascaris suum* extract and concanavalin A

Eight-week-old male BALB/c mice were housed in the animal care facility at the Keizo Asami Immunopathology Laboratory, Federal University of Pernambuco (LIKA/UFPE). Extract of adult *A. suum* worms was obtained in according to Souza et al. (2002). Concanavalin A type IV (ConA) (Sigma-Aldrich, St. Louis, MO, USA) was diluted in sterile 0.01 M PBS at pH 7.2.

Experimental groups

Three experimental groups were formed (6 animals/group). EAH was induced in the mice by intravenously administering 20 mg/kg of ConA diluted in sterile PBS (2 mg/mL). After two hours, some of these animals received PBS (i.p.) (EAH group) and the remainder of the animals received 1 mg of ASC (i.p.) (EAH+ASC group). The control animals received only PBS (control group). The experimental model and hepatitis parameters were adjusted as described by Nascimento et al. (2014). This protocol was approved by the Ethics Committee of the Biological Sciences Research Center, UFPE (no. 23076.041797/2013-40).

Detection of anti-*Ascaris* antibodies using ELISA

Blood samples were collected 8 h, 24 h and 7 days after induction of EAH. *Ascaris*-specific IgG1 and IgG2a antibodies were titrated using ASC (5 µg/mL)-coated 96-well plates (Nunc MaxiSorp, Denmark) and biotinylated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates Inc, USA). The reactions were developed using a streptavidin-HRP conjugate (Sigma-Aldrich, USA) and an O-phenylenediamine (Sigma-Aldrich, USA) solution in 0.1 M citrate buffer plus H_2O_2. The plates were read in 450 nm. The results were expressed as the mean of optical density ± standard deviation at a dilution corresponding to the linear part of the titration curve (1:10).

Culturing of spleen cells and TGF-β measurement using ELISA

Seven days after induction of EAH, the spleens of the mice in the EAH and EAH+ASC groups were harvested. Cell suspensions were prepared as described by Nascimento et al. (2014) and the supernatants were harvested (72h) and assayed for TGF-β content using a commercial kit (Invitrogen, Novex, USA).

Statistical analysis

For antibody production analysis, the two-way ANOVA test was used to evaluate the differences among groups. Multiple comparisons were performed using the Bonferroni test. For TGF-β production analysis, the t test was used. For statistical analyses, we used GraphPad Prism 5.0 (GraphPad Software, San Diego, USA), and all findings were considered significant at $p < 0.05$. All the procedures were repeated three times to evaluate the reproducibility of the results, and one representative of the three independent analyses was presented.

Results

In the EAH group, the levels of anti-*Ascaris* IgG1 and IgG2a were extremely low and followed the same kinetics, with much lower levels at 24 h. In the EAH+ASC group, production of IgG2a only occurred at the 7th day and was at higher levels than in the EAH group. Regarding anti-*Ascaris* IgG1, production occurred at 8 h, 24 h and the 7th day, but was significantly greater at the latter two times (Figure 1A), in relation to EAH group. In the control group, there were no detection of anti-*Ascaris* antibodies (data not shown).

TGF-β was measured in the supernatant from culturing of splenocytes only with medium and stimulated with ConA or ASC. Under these conditions, the TGF-β production was greater in the EAH+ASC group than in the EAH group (Figure 1B).

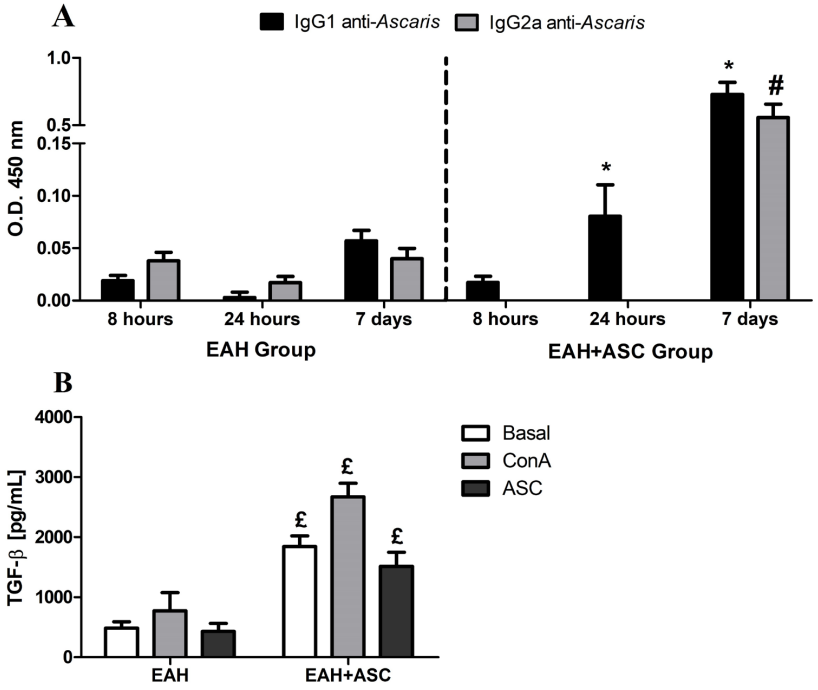

Figure 1. Anti-*Ascaris* IgG1 and IgG2a isotypes (A) in the plasma of BALB/c mice, 8 h, 24 h and 7 days after induction of experimental autoimmune hepatitis (EAH) and treatment with a soluble extract *Ascaris suum* worms (ASC), by ELISA. TGF-β levels (B) in cultures of splenocytes from the EAH and EAH+ASC groups (72 h; 6 x 10^6 cells/mL), 7 days after induction of EAH, that were cultured only with medium (Baseline), ConA (10 ng/mL) or ASC (20 µg/mL). The results are presented as the mean ± standard deviation of 6 animals/group. *p<0.05 in relation to the EAH group for IgG1; #p<0.05 in relation to the EAH group for IgG2a; £p<0.05 in relation to the EAH group for TGF-β.

Discussion

Administration of an extract of *A. suum* was previously found to attenuate the hepatic damage in EAH through deviating the Th1 immune response to a Th2/IL-10 pattern (Nascimento et al., 2014). Here, it was possible to demonstrate that treatment with ASC induced elevated levels of TGF-β and anti-*Ascaris* IgG1 and IgG2a, although IgG1 was produced earlier and at higher levels.

In the EAH group, production of specific anti-*Ascaris* antibodies was observed. This can be explained by the administration of ConA. It gives rise to polyclonal expansion of T lymphocytes and consequently, B lymphocytes (Tiegs et al., 1992). In the EAH+ASC group, the kinetics of production of the IgG1 and IgG2a isotypes were distinct. Production of the IgG2a isotype was only detected at the 7th day. On the other hand, production of IgG1 already occurred at higher levels at 24 h and was also seen at the 7th day. This result corroborates the previous description of redirecting of the immune response in EAH caused by ASC, to a Th2 profile, within the first 24 hours. It is known the IgG1 class-switching IL-4 and Asc-dependent (Silva et al., 2006). Indeed, the production of the IgG1 began together with higher levels of IL-4 and IL-13 (Nascimento et al., 2014).

The relationship between IgG-FcγR and IL-10 synthesis via macrophages is known in experimental parasite model (Buxbaum & Scott, 2005; Fairfax et al., 2012). Helminth antigen induced a hepatic microenvironment with high levels of IL-4 and IL-13 may promote differentiation of macrophages that produce IL-10, named M2 that are relevant in the tissue repair (Rolot & Dewals, 2018). In EAH, ASC caused increases in IL-4, IL-10 and IL-13 levels (Nascimento et al., 2014). IL-10 is crucial for tolerance induction in EAH (Erhardt et al., 2007). It is possible that deviation of production of IgG isotype to the subclass IgG1, in response to ASC, may be negatively modulating the lesion in EAH through induction of IL-10 by macrophages at an early stage (24 h). Tests using FcγR knockout mice might help to confirm this hypothesis.

In addition to production of IL-10 via IgG1, it is possible that the suppression mediated by ASC in EAH may also occur through induction of TGF-β. Blockage of TGF-β suppresses the functioning of $CD4^+CD25^+Foxp3^+$ T lymphocytes, thus making mice more susceptible to EAH (Wei et al., 2008). Here, treatment with ASC induced elevated levels of TGF-β. However, inflammatory cytokines (IL-17, IL-22) deserve to be studied.

Conclusions

In conclusion, higher levels of IgG1 and TGF-β may indicate control over the Th1 response during EAH. However, further studies are needed to confirm this hypothesis. Comprehension of the modulatory mechanisms, identification and characterization of the molecules involved in this process may assist in developing alternative economical therapies for treating inflammatory diseases, such as synthesis of analogues of *A. suum* that stimulate immunological tolerance.

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