Expression of SmATPDase 1 and SmATPDase 2 in Schistosoma mansoni eggs favors IL-10 mediated immune system modulation in infected individuals.

Thalisson Artur Ribeiro Gomides
Universidade Federal de Ouro Preto

Márcio Luís Moreira Souza
Universidade Federal de Juiz de Fora

Amanda Braga Figueiredo
Camargo Cancer Center

Marlucy Rodrigues Lima
Universidade Vale do Rio Doce

Alda Maria Soares Silveira
Universidade Federal de Juiz de Fora

Girley Francisco Machado Assis
Universidade Federal de Juiz de Fora

Lucia Alves de Oliveira Fraga
Universidade Federal de Juiz de Fora

Gabriela Silveira-Nunes
Universidade Federal de Juiz de Fora

Leticia Martucci
Universidade Federal de Juiz de Fora

Jennifer Delgado Garcia
Universidade Federal de Juiz de Fora

Luís Carlos Crocco Afonso
Universidade Federal de Ouro Preto

Andréa Teixeira-Carvalho
Instituto René Rachou, Fundação Oswaldo Cruz - FIOCRUZ

Pauline Martins Leite (✉ pauline.leite@ufjf.br)
Universidade Federal de Juiz de Fora

Research Article

Keywords: Schistosomiasis, SmATPDases, IL-10, immunomodulation, cytokines

Posted Date: March 9th, 2023
Abstract

Background

Schistosomiasis is a chronic disease that affects over 200 million people worldwide. A pivotal role of IL-10 is down-regulating Th1 and Th2 responses to schistosome antigens, which can favor the parasite establishment. The SmATPDases degrade ATP and ADP in AMP and adenosine, a molecule with anti-inflammatory properties. We evaluated the expression of SmATPDases 1 and 2 enzymes in *S. mansoni* eggs obtained from infected individuals as a possible parasite-related factor that could influence the host immune response and the clinical outcome of the disease.

Methods

Fecal samples were collected from 40 infected individuals to detect coding regions of the enzymes by the qPCR. The production of cytokines was measured in supernatants of PBMC cultures. The analysis was performed by the global median determination for each cytokine and set up high producers (HP) of cytokines.

Results

Six individuals expressed SmATPDase 1 in their fecal samples, 6 expressed SmATPDase 2, and 6 expressed both enzymes. The group who expressed only SmATPDase 1 showed a high frequency of IFN-γ, TNF, IL-4 HP, and a low frequency of IL-6 HP. The group who expressed only SmATPDase 2 showed a high frequency of IFN-γ, IL-6, and IL-4 HP and a low frequency of IL-10 HP. The group who expressed both enzymes showed a high frequency of IL-10 HP and low frequencies of IFN-γ, IL-6, IL-2, IL-4, and IL-13 HP. In the group that had SmATPDase 2 expression was observed higher indices the ratio between IFN-γ/IL-10 than individuals that showed expression both enzymes. The positive correlation between infection intensity and IL-10 levels remained only in the positive SmATPDase group. Overall, the analysis revealed that 62.5% of the cytokines presented reduced frequency in the group of individuals expressing both enzymes, the IL-10 is the only cytokine induced by the expression of both enzymes and the expression profile of SmATPDases is relevant data for grouping individuals.

Conclusions

The expression of both enzymes in the parasite's eggs seems to be a new undescribed factor that negatively modulates the host immune response by inducing high IL-10 production, which, in turn, can contribute to the survival of the parasite.

Background
Schistosomiasis is an important parasitic disease that affects more than 200 million people worldwide [1–3]. It is estimated that 1.5 million people in Brazil live in areas at risk of infection. Despite the reduction in schistosomiasis cases in recent years, it remains a relevant disease for public health and can mainly affect impoverished people living in rural areas [4–8].

In the course of infection, the immune response is characterized by a Th1 response inflammatory (IL-6, TNF, IFN-γ, IL-2) induced by worm antigens, Th2 response (IL-4 and IL-13) by an egg-driven and immunomodulatory response (IL-10) [9–11].

The parasite possesses several mechanisms for interfering with the normal functioning of the host immune system, such as cytokine production [12]. In this context, the regulatory cytokine IL-10 is strongly associated with the immunomodulatory profile triggered along with the chronic infection [1, 13]. During this phase of infection, a high parasitic burden seems to influence the increase of IL-10 to control the immune response during the infection [14].

Several molecules have been identified as candidates for *S. mansoni* virulence factors, which may contribute to schistosomiasis's pathogenicity. Among them, SmATPDase, smAP, and smPDE have been considered possible candidates since they modulate the host's immune system [15]. ATP-diphosphohydrolases (EC 3.6.1.5) hydrolase a variety of nucleoside tri-and diphosphates and show five conserved domains motifs [16,17]. The presence of enzymes has been described in a wide range of eukaryotic organisms and their enzymatic activities appear to be related to the virulence of parasites [18,19], regulation of inflammatory responses [20], thrombus regulation in humans [21] and as participants in the escape of the parasite from the host immune system, through eventual blocking of platelet activation [17,22]. In the extracellular environment, ATP is a potent pro-inflammatory mediator, which promotes immune cell activation and chemotaxis to the location of the worms. On the other hand, adenosine generated by the dephosphorylation of ATP, downmodulates the inflammatory response by increasing the intracellular cAMP concentration [23–25].

The activity of these enzymes in *Leishmania amazonensis* seems important for the parasite's survival in macrophages [26]. In addition, a positive association between the ectonucleotidase activity and the development of severe clinical forms of cutaneous leishmaniasis was evidenced [18].

In *S. mansoni*, a broad expression of ATP diphosphohydrolase at all stages of the parasite life cycle has been described [27]. Studies have suggested that *S. mansoni* ATP diphosphohydrolase isoforms may contribute to the parasite's ability to minimize attack and the risk of thrombosis, as well, modulate the host immune responses or stimulate IL-10 synthesis [20, 28–30].

Since ecto-nucleotidases have a crucial role in the metabolism of extracellular nucleotides, which, in turn, can be correlated to the host immune response and development of infection, we focused our study on analyzing the expression of SmATPDase 1 and SmATPDase 2 enzymes in *S. mansoni* eggs obtained from infected individuals as a possible parasite-related factor that could influence the host immune response, and consequently the clinical outcome of the disease. Our pioneer study results showed that the expression of
SmATPDases in *S.mansonii* eggs seems to negatively modulate the host immune response by IL-10 induction.

**Methods**

**Study Population**

This study was conducted in Chonin de Baixo, a rural district of Governador Valadares -MG, Brazil, with about 1,083 inhabitants from November 2015 to August 2017. This district is endemic for schistosomiasis and is part of a region characterized by several water sources that are used for the leisure activities of the local population [31,32]. Of these 1,083 individuals, 830 performed the feces examination to detect eggs of the *S. mansoni* parasite through a partnership with the Zoonosis Division from the Municipal Secretariat of Health of Governador Valadares as recommended by Schistosomiasis Control Program (PCE) [5], resulting in 62 individuals infected with *S. mansoni*, which means a prevalence of 74.7 cases/1,000 inhabitants (Fig. 1). Out of the individuals infected, 40 subjects provided the required three fecal samples for parasitological examinations by Kato-Katz and spontaneous sedimentation (HPJ) methods and blood collection to quantify cytokines. In addition, the molecular evaluation for the expression of enzymes in *S. mansoni* eggs was performed in fecal samples. The classification of individuals as uninfected was based on negative results in parasitological and constitutive gene expression (eukaryotic translation initiation factor 4E - eIF4E). On the other hand, the individuals who obtained positive results for at least one of the parasitological methods and/or constitutive expression gene were classified as positive. The study design was based on judgmental sampling using a non-probabilistic approach. Thus, from this classification, the study participants were organized into two groups: 1) uninfected control group composed of 13 negative individuals for all evaluations, living in areas at risk for *S. mansoni* infection, Chonin de Baixo and Governador Valadares; 2) infected group, composed of 40 individuals who presented positive *S. mansoni* tests from Chonin de Baixo, who scored all evaluations (Fig. 1).

**Parasitological Analysis**

Schistosomiasis was diagnosed using HPJ and Kato-Katz methods [33,34]. The intensity of infection was calculated as described by [33].

**Extraction of *S. mansoni* eggs**

The *S. mansoni* eggs were obtained after the HPJ technique. The homogenate containing the eggs was passed through a series of sieves (300–180 µm), and the eggs were collected by sedimentation and cleaned by washing them six times in sterile phosphate-buffered saline. The eggs were resuspended in RNA later (Sigma-Aldrich) and stored at -80°C for further RNA extraction [35].

**RNA extraction and qPCR**

The feces samples containing the eggs in later RNA were ground by the use of a tissue homogenizer (POLYTRON), and after maceration, the samples were incubated for 5 minutes at room temperature to ensure complete nucleoprotein dissociation. The RNA was extracted using the commercial QIAamp® viral RNA Mini
Kit, following the manufacturer’s instructions (Qiagen GmbH, Hilden, Germany). All extraction steps were performed at room temperature. Thus, the eluted RNA was read at 260 and 280 nm in the spectrophotometer and quantified from the following equation: Concentration (µg / µL) = A260 x 40 x dilution / 1000. The RNA was stored in a freezer at −80°C until the reverse transcription was performed.

After RNA isolation, the cDNA was synthesized using 2 µg of total RNA, a primer oligo (dT) 20, and Superscript III RT (Invitrogen, CA). Gene expression was measured by quantitative real-time PCR (qPCR) using SYBR green amplification systems. The targets for the amplification are the gene regions specific for *S. mansoni*, as the endogenous control eIF4E forward 5’-TGTTCCAACCACGGTCTCG-3’ and reverse 5’-TCGCTTCCAATGCTTAGG-3’ [35,36, 37]; SmATPDase1 forward 5’-CTGATGCCGTTATGAAGCTTGGAC-3’ and reverse 5’-GCAGTAAA CCCTTGGTGAGTAATTTTGGCA-3’, SmATPDase2 forward 5’-GGTTATGGATTCCCGGTCCGAGATA-3’ and reverse 5’-TGAAAATAAGGCACCAAGACTCCCAA-3’ [29].

The expression of gene regions specific to SmATPDase 1 and SmATPDase 2 was considered positive only when there was the concomitant expression of the endogenous control eIF4E. The number of cycles required for the fluorescent signal to cross the threshold (Ct) ≤ 37 were considered positive reactions for the genes investigated. The schistosome eIF4E has 32% identity and 51% similarity with human eIF4E. Notably, schistosome eIF4E is more highly divergent from human eIF4E than nematode eIF4Es [36]. Additionally, the eIF4E gene increases the amplification efficiency (98.8%), ensuring high sensitivity [38].

**Cell Culture and Cytokine Measurements**

PBMC were purified from a collection of 20 mL of heparinized venous blood samples following the procedure described by [39]. PBMC were incubated after stimulation with 25µg/mL soluble *S.mansoni* egg antigen (SEA) in an incubator at 37°C in an atmosphere containing 5% CO2. The supernatant was collected after 72 hours of incubation and maintained at -70°C for measurement of cytokines by flow cytometry (BD FACSVerse™). Levels of cytokines were determined by the Cytometric Bead Array Assay (Becton Dickinson Biosciences Pharmingen, San Diego, USA). Data were analyzed with the aid of the software BD FCAP Array 3.0 (Becton Dickinson, USA). The results were expressed in Mean Fluorescence Intensity (MFI) and index values (Ag stimulated culture – SEA / unstimulated culture - CC).

**Cytokine signature**

The index values of each cytokine (SEA-Ag / control - CC) were taken into account for the analysis of the cytokine in the supernatant of PBMC culture. Cytokine profile was assessed to identify individuals with low (≤ global median) and high (> global median) production using the global median value of each cytokine as a cut-off. The whole universe of data obtained for the groups was considered for calculating the global median. The overall median for each cytokine was calculated (TNF = 4.62, IL-6 = 1.67, IFN-γ = 2.73, IL-2 = 1.19, IL-4 = 1.17, IL-13 = 3.64 and IL-10 = 7.05) and these values were used as the cut-off to classify the individuals as being a high (HP) or low (LP) cytokine producer (Supplementary Fig. 1). This strategy allows multiple analyzes between groups, as shown by [40–42].

**Radar charts**
In radar charts, each axis represents the percentage (%) of individuals showing high production for each cytokine. The values of each axis are connected to form a central polygonal area representing the cytokine’s global balance. An increase or decrease of the central polygonal area reflects either a higher or a lower contribution of cytokine profile for each SmATPase expression group. Only those groups that presented more than 50% of individuals as high cytokine producers were considered relevant.

**Multivariate Statistical Analysis**

Multiple Correspondence Analysis (MCA) is an unsupervised method and a data reduction technique that allows the major sources of variation in a multi-dimensional dataset to be analyzed without introducing inherent bias. MCA analysis was performed to examine any intrinsic variation in the classification of individuals regarding the categorized variables cytokine production, expression of SmATPases, laboratory data (parasite load), and sociodemographic data (age and gender) and to see if any grouping was formed. The first principal component (PC) is a linear combination of the original variables that incorporates the most significant variation sources within a dataset. The second and subsequent PCs are different latent variables that explain the most significant sources of variation leftover beyond the first PC and lie orthogonal to it. The variation in this dataset using MCA indicates the participation of each variable presented by the individuals in their position in the bidimensional plane. In addition, MCA allows us to observe which variables contributed to the grouping of individuals.

**Statistical analysis**

The data were analyzed by GraphPad, Prism 5.0 software (La Jolla, CA, USA). Analyzes were made using the Mann-Whitney test and Spearman Correlation. Additional analysis was carried out using the Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). The differences were considered significant at the 0.05 level. The MCA was determined in the R program using the FactoMineR package.

**Results**

**Study population**

This study involved 13 non-infected control individuals and 40 individuals infected by *S. mansoni*. Information concerning age, gender, and infection level are provided in Table 1. Among the 40 individuals, 22 (55.0%) were males, and 18 (45.0%) were females, and their ages ranged from 7 to 73 years, with a mean age of 32.9 ± 19.6 and a median of 26 years. The infection levels ranged from 0 to 648 epg (eggs per gram) of feces. Detailed data are shown in Table 1. The ultrasound examinations were conducted and all participants showed no signs of periportal fibrosis.
Table 1
Characterization of the study group

<table>
<thead>
<tr>
<th>Parameters</th>
<th>S. mansoni-Infected</th>
<th>Uninfected Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>40.0 (75.5%)</td>
<td>13.0 (24.5%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>7.0–73.0</td>
<td>21.0–73.0</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>32.9 (± 19.6)</td>
<td>35.2 (± 15.6)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>26.5 (15.5–52.7)</td>
<td>35.0 (21.5–40.5)</td>
</tr>
<tr>
<td>Age Group - n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.0 (20.0)</td>
<td>0.0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>15.0 to 30.0</td>
<td>15.0 (37.5)</td>
<td>5.0 (38.5)</td>
</tr>
<tr>
<td>31.0 to 60.0</td>
<td>11.0 (27.5)</td>
<td>7.0 (53.8)</td>
</tr>
<tr>
<td>&gt; 60.0</td>
<td>6.0 (15.0)</td>
<td>1.0 (7.7)</td>
</tr>
<tr>
<td>Gender - n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22.0 (55.0)</td>
<td>7.0 (53.8)</td>
</tr>
<tr>
<td>Female</td>
<td>18.0 (45.0)</td>
<td>6.0 (46.2)</td>
</tr>
<tr>
<td>Egg Counts (Eggs/g of Feces)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>72.1 (± 147.8)</td>
<td>0.0 (± 0.0)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>7.0 (0.0–55.5)</td>
<td>1.0 (0.0)</td>
</tr>
<tr>
<td>Egg distribution (eggs/g of feces) – n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.0 (72.5)</td>
<td>0.0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>50.0–100.0</td>
<td>4.0 (10.0)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>&gt; 100.0</td>
<td>7.0 (17.5)</td>
<td>0.0 (0.0)</td>
</tr>
</tbody>
</table>

SD – Standard deviation IRQ – Interquartile Range

Levels of cytokines produced by PBMC culture

The Fig. 2 shows the median levels of TNF, IL-6, IFN-γ, IL-2, IL-4, IL-13 and IL-10 cytokines. Similar results were observed in the group of uninfected individuals (data not shown).

Expression of SmATPDases in fecal samples from S. mansoni-infected individuals

The results involving the expression of SmATPDase 1 and SmATPDase 2 will be related to 39 (one individual was deleted due to technical issues) infected individuals. Table 2 shows that expression of at least one of the ectonucleotidases (Positive SmATPDase) was detected in stool samples from 18 (46.2%) S. mansoni-
infected individuals. Of these, 6 (15.4%) expressed only the SmATPDase 1 enzyme, 6 (15.4%) expressed only SmATPDase 2, and the other 6 (15.4%) expressed both SmATPDase 1 and SmATPDase 2. On the other hand, it was not possible to identify SmATPDase 1 and/or SmATPDase 2 expression in fecal samples of 21 (53.8%) individuals (Undetectable SmATPDase).

Table 2
Characterization of the study group according to the expression of SmATPDases in fecal samples from *S. mansoni*-infected individuals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Positive SmATPDase (n = 18.0)</th>
<th>Undetectable SmATPDase (n = 21.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SmATPDase 1</td>
<td>SmATPDase 2</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>14.0–66.0</td>
<td>9.0–73.0</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>36.2 (± 24.3)</td>
<td>44.0 (± 26.4)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>34.5 (14.0–57.0)</td>
<td>49.0 (17.2–66.2)</td>
</tr>
<tr>
<td>Egg Counts (Eggs/g of Feces)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.0–12.0</td>
<td>0.0–356.0</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>4.0 (± 5.1)</td>
<td>77.3 (± 138.3)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>0.0 (0.0–9.0)</td>
<td>30.0 (0.0–125.0)</td>
</tr>
<tr>
<td>Egg distribution (Eggs/g of Feces) – n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50.0</td>
<td>6.0 (100.0)</td>
<td>5.0 (83.3)</td>
</tr>
<tr>
<td>50.0–100.0</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>&gt; 100.0</td>
<td>0.0 (0.0)</td>
<td>1.0 (16.7)</td>
</tr>
</tbody>
</table>

SD – Standard deviation IQR – Interquartile Range

The age, parasite load, and infection intensity of the individuals expressing the SmATPDases in stool samples are described in Table 2.

**Expression of SmATPDases alter the cytokine profile in infected individuals**
The panoramic profile of cytokine is presented in Fig. 3. To understand better the influence of SmATPases expression on the immune response of individuals, the HP frequencies presented by individuals who did not show expression of SmATPases in their stool samples (Undetectable SmATPase) (Fig. 3a) was used to construct an ascendant reference curve to compare the study groups (Fig. 3b, 3c, 3d e 3e).

We observed that the group who expressed only SmATPase 1 showed a high frequency of IFN-γ, TNF, and IL-4 high producers (HP) and a low frequency of IL-6 high producers (HP) (Fig. 3b). The group who expressed only SmATPase 2 showed a high frequency of IFN-γ, IL-6, and IL-4 HP and a low frequency of IL-10 HP (Fig. 3c). Interestingly, the group who expressed both enzymes showed a high frequency of IL-10 HP and low frequencies of IFN-γ, IL-6, IL-2, IL-13 and IL-4 HP. (Fig. 3d). The group of uninfected individuals showed a high frequency of IFN-γ and IL-6 HP and a low frequency of IL-2, IL-13, IL-4, and IL-10 HP (Fig. 3e). We considered substantial change when the proportion of subjects above the cut-off shifted from quartile to more or fewer positions.

Our results show that the frequency of HP individuals to IFN-γ was elevated in the group that showed expression of SmATPase 2 and reduced in the group that showed expression of both enzymes. Differently, the frequency of HP individuals to IL-10 was elevated in the group that showed expression of both enzymes and reduced in the group that showed expression of SmATPase 2. Thus, the comparison of the IFN-γ / IL-10 ratio presented higher indices in the group who had SmATPase 2 expression than those who had the expression of both enzymes (p < 0,05) (Fig. 4), suggesting that IL-10 may be controlling the IFN-γ production.

Modulating effect of the expression of SmATPase 1 and SmATPase 2 dependent on IL-10

To further characterize the cytokine pattern of infected individuals according to the distinct expression enzymes groups, we have constructed radar charts and a Venn diagram (Fig. 5). A relevant difference in the global cytokine profile was observed in infected individuals who expressed SmATPase (SmATPase 1, SmATPase 2, and both enzymes) in their stool samples than to those that did not present expression of the enzymes (Undetectable SmATPase). In addition, the radar chart revealed that 5/7 (71.4%) of the cytokines were highly induced by the expression of the SmATPase 1 enzyme, 4/7 (57.1%) of the cytokines were highly induced by the expression of the SmATPase 2 enzyme, while 5/7 (71.4%) of the analyzed cytokines presented reduced frequency in the group of individuals expressing both enzymes in stool samples. Interestingly, in this group, only for IL-10, a frequency of over 50% of high-producing individuals was observed. In addition, Venn diagram analysis showed that two cytokines (TNF and IL-2) were induced exclusively by SmATPase 1 expression, one cytokine (IL-6) was induced exclusively by SmATPase 2, and three cytokines (IFN-γ, IL-4, and IL-13) were induced by both SmATPase 1 and SmATPase 2 expression. IL-10 is the only cytokine induced by the expression of both enzymes. This finding suggests a possible modulatory effect on the immune system when SmATPase 1 and SmATPase 2 are expressed concomitantly, probably due to the modulatory effects of IL-10.

Expression of SmATPases contributes to the positive association between infection intensity and IL-10 index
Intending to evaluate the contribution of parasite load on IL-10 production by individuals showing both enzymes (SmATPDase 1 and SmATPDase 2) expression in parasite eggs, we analyzed the association between infection intensity and IL-10 index. We observed a positive correlation between the IL-10 index and the parasite load in the infected population (Fig. 6). This correlation was significant when were considered all individuals, independent of the expression of the enzyme in the parasite eggs. Interestingly, when analyzing the group of individuals who showed expression of at least one of the enzymes, a positive correlation was observed. This same correlation was not observed among individuals who did not have S.mansoni eggs expressing the enzymes. In addition, in the positive SmATPDase group, we observed that subjects expressing both enzymes in their feces are among the individuals with the highest intensities of infection (> 100 epg) and highest IL-10 rates.

Profile of SmATPDases expression in the stool samples of the S.mansoni infected individuals is shown to be relevant data to group the individuals in the multivariate analysis

To better clarify the role of the various factors in the immunological response, the variables related to the characterization of the individuals considering age, sex, the intensity of infection, classification of individuals regarding cytokine production, and the presence or absence of SmATPDase expression in S.mansoni eggs were used (Fig. 7). The values obtained through the set of variables discussed allow us to analyze the profiles so that the greater the proximity between the points represented in the multi-dimensional graph, the greater the similarity between the individuals participating in the present study. To optimize the graphical representation of this multi-dimensional analysis, we used the two-dimensional Cartesian plane that contemplates 37.34% of the total variability of the data (22.10% and 15.24% in the first and second dimensions, respectively). The table reports (Fig. 7b) show how each of the variables contributes to the explanation of the total variability of the samples in dimension one and dimension two. Thus, IL-13 alone can explain 24.60% of the variabilities of the samples in dimension one, followed by IL-4, IL-2, IFN-γ, SmATPDase Group, IL-6, Parasite Load, Gender, IL-10, Age and TNF. A variable TNF alone can explain 28.60% of the variabilities of the samples in dimension two, followed by IL-10, SmATPDase Group, Parasite Load, IL-6, Gender, IFN-γ, IL-4, Age, IL-2 e IL-13.

In Fig. 7a, it was possible to identify an organization pattern of the analyzed cytokines so that the high and low categories were arranged symmetrically in both dimensions of the Cartesian plane, perceived by their quadrants. Thus, being classified as a high producer of cytokines leads to the positioning of individuals in the upper (first and second) quadrants, while being classified as a low producer favors their positioning in the lower quadrants (third and fourth).

The expression of SmATPDases proved to be one of the contributing factors to the disposition of the individuals in the Cartesian plane. SmATPDase 1 expression is associated with high producers of various cytokines, such as TNF, IFN-γ, IL-4, and IL-2 (second quadrant). SmATPDase 2 expression appears to be associated with the classification as a high producer of IL-6 and low producer of IL-10 and TNF (third quadrant), a similar behavior to the undetectable SmATPDase group.

Interestingly, the expression of both enzymes seems to be associated with a differentiated immune profile, associating with the classification of individuals as high producers of IL-10 and low producers of most
cytokines (first and fourth quadrants). In Fig. 7c, the group of individuals in the Cartesian plane is highlighted, considering the expression profile of the SmATPDases was considered. All individuals presenting SmATPDase 1 expression in *S. mansoni* eggs are in the first or second quadrant, four of the six individuals with SmATPDase 2 expression in *S. mansoni* eggs are in the second and third quadrant, and five of the six individuals presenting the expression of both enzymes in *S. mansoni* eggs are grouped in the fourth and first quadrant. Thus, the expression profile of SmATPDases in the fecal samples of the infected individuals is shown as relevant data for the grouping of individuals through multivariate analyzes (Fig. 7d).

**Discussion**

The main goal of the present study was to evaluate the role of the expression of SmATPDase 1 and SmATPDase 2 enzymes in *S. mansoni* eggs on the immune response of infected individuals living in low-endemicity areas. The study area matches the epidemiological situation in many endemic Brazilian regions, where frequent treatment cycles have reduced clinical cases and morbidity considerably and decreased individual and community parasite loads [43].

Table 1 shows that among the 40 individuals infected with *S. mansoni*, 55.0% were males, 37.5% were between 15 and 30 years old, and 72.5% exhibited infection levels lower than 50 epg of feces. The authors [44, 45] demonstrated highest infection rate in children and young adults and also the most infected individuals were male. More effective immune responses might explain reduced parasite loads in elderly individuals, reduced reinfection rates, reduced exposure due to altered habits and/or aging worms, and reduced fertility of female parasites [46, 47]. Considering that water contact is a risk factor for *S. mansoni* infection [48], our results can be explained since that was described as a greater exposure of males engage in leisure activities with water contact (data not shown).

During their development in the human body, the different parasitic stages of schistosomes induce significant alterations in the immune response [49, 50]. The relation of the Th1 cytokines, IL-2, and IFN-γ during the acute phase mediate the establishment of early granulomas [51]. IL-4 and IL-10 downregulate the Th1 response during the early stage of schistosomiasis, and cytokine Th1-type polarization can lead to 100% mortality during acute illness [52]. In humans, IFN-γ has a protective role in controlling severe fibrosis, so low levels of IFN-γ and high levels of TNF-α, IL-4, IL-5, IL-10, and IL-13 have been associated with an increased risk of developing severe liver fibrosis [53, 54]. On the other hand, IL-10 also plays a role in the modulation of the inflammatory process and prevention of more severe forms of the disease [55]. Thus, the role of these T helper cell subpopulations on the human immune response to infection by *S. mansoni* has not yet been well established. We demonstrate that the stimulation of PBMC with SEA increases cytokine levels in the infected or uninfected individuals (Fig. 2). Previous studies already demonstrate that stimulation with schistosome antigens alters the immune response of infected individuals and uninfected residents in area endemic [39, 56, 57]. Immune sensitization of naturally resistant individuals in the endemic area might occur for several reasons: maternal-fetal interaction (idiotypes and antigens), single-sex infections, aborted infection before worm maturation, light infection (difficult to detect by stool examination), an anti-fecundity response or self-cure [58–61].
Ectonucleotidases correlate with the infectivity of *S. mansoni* parasites since they play a role in the escape from host defenses through platelet activation [27, 62]. In addition, drugs that inhibit the activity of these enzymes are considered schistosomicides [63]. The correlation between ectonucleotidases and virulence has been observed among parasites from different species [18, 64–66]. These enzymes are present during all stages of the *S. mansoni* life cycle [27, 67]. In our investigations, it was possible to identify SmATPDases expression in feces samples of 46.2% of infected individuals (SmATPDase 1, SmATPDase 2, or Both Enzymes) (Table 2). In addition, the frequencies of individuals expressing SmATPDase 1 and SmATPDase 2 enzymes in stool samples were similar (15.4%). Recent studies claim that the expression of these enzymes in *S. mansoni* eggs neutralizes ATP-associated molecular damage-mediated inflammatory signaling (DAMPs) and limits the host’s attempts to concentrate inflammatory mediators around worms [29]. Thus, the expression of *S. mansoni* ATP-diphosphohydrolases helps to decrease the host’s immune defenses and promote parasite survival. However, the effect of the expression of these enzymes on the immune response in humans is currently unknown. For this reason, our research group seeks to identify changes in the cytokines profile caused by the expression of these SmATPDases (Figs. 3 and 5). The panoramic profile of cytokines was performed for all groups (Infected -Undetectable SmATPDase, SmATPDase 1, SmATPDase 2, and Both Enzymes and Uninfected control group). The uninfected individuals showed a high frequency of IFN-γ and IL-6 HP and a low frequency of IL-2, IL-13, IL-4, and IL-10 HP. The cytokine profile in the undetectable SmATPDase group was characterized by a slight increase in the frequency of high-cytokine producer individuals in the Th2 and regulatory profile (IL-13, IL-4, and IL-10). This profile is characteristic in schistosomiasis since, after egg deposition, the Th2 response becomes more evident with the production of cytokines such as IL-4, IL-5, IL-10, and IL-13 [10, 11]. However, the expression of enzymes in the parasite's eggs can alter this immune profile after oviposition. The expression of SmATPDase 1 alone was related to an increase in the frequency of high producers individuals by IFN-γ, TNF, and IL-4. The SmATPDase 2 expression alone was associated with an increase in the frequency of high producers individuals of IFN-γ, IL-6, and IL-4. The role of adenosine in cellular receptors is associated with its concentration available in the extracellular environment. A smaller amount of adenosine will act preferentially on pro-inflammatory receptors (A1 and A3), while a greater amount will act on the immune response inhibiting receptors [68, 69]. Our results suggest that adenosine production in samples expressing only one of the enzymes may favor a pro-inflammatory response. Previous results [70] showed a possible existence of opposing effects involving Fc-gamma receptor-associated adenosine A1 and A2 receptors on mononuclear phagocytes. These authors verified that low adenosine concentrations lead to a pro-inflammatory response via the A1 receptor. Another interesting fact in our results is the expression of SmATPDase 1 and SmATPDase 2, favoring the greater cytokine production of the Th1 and Th2 profiles concomitantly (IFN-γ and IL-4). Results are shown by [71] identifying IFN-γ + IL-4 + cells in mice infected with *S. mansoni*, cytokines of the Th1 and Th2 profiles, probably to regulate the development of fibrosis related to an extended Th2 immune response. The cytokine IL-10 modulates the immune response in chronic asymptomatic patients, which could be an important factor in controlling schistosomiasis morbidity [14, 72]. According to our results in the SmATPDase 1 and SmATPDase 2 expression only, in which there was an increase in the cytokine frequencies of the Th1 and Th2 profiles, the frequency of IL-10-high producers individuals was lower than 50% (Fig. 3b and c and Fig. 5). We observed that SmATPDase 1 and SmATPDase 2 expression concomitantly promotes the frequency of IL-10 high producers individuals above 50% and decreases the frequency of IFN-γ, IL-6, IL-2, IL-13, and IL-4- high
producers individuals. Thus, the individuals expressing both enzymes in fecal samples demonstrated a negative modulation mediated by IL-10 (Fig. 3d). This modulating effect becomes more evident when we compare the radar graphs (Fig. 5) and the IFN-γ / IL-10 graph (Fig. 4). Possible associations between adenosine and IL-10 production have already been demonstrated in regulating the immune response [73–75].

Due to the possible immunomodulatory effect of *S. mansoni* ATP-diphosphohydrolases *in vitro*, these enzymes have been considered promising molecules for developing new drug candidates for the treatment of schistosomiasis [76]. Our results showed a positive correlation between parasite load and IL-10 levels in the study population (Fig. 6A). However, when organizing the individuals based on the expression of the enzymes (Positive SmATPDase/ Undetectable SmATPDase), this correlation was only observed in the SmATPDase (Positive SmATPDase) expression group (Fig. 6). In addition, we observed that three (50%) individuals expressing both enzymes in their feces samples had a high parasitic load (> 100 epg) and high levels of IL-10 (Fig. 6). Thus, the increase of IL-10 in individuals with high parasitic load may be influenced by the expression of both enzymes concomitantly. The MCA results revealed that SmATPDase expression in fecal samples of infected individuals is relevant data for categorizing individuals, allowing the association of the expression of these enzymes with sociodemographic, parasitological, and immunological data. In addition, this analysis showed that the concomitant expression of both enzymes (SmATPDase 1 and SmATPDase 2) has an immunomodulatory effect in the infected individuals, contributing to a significant reduction in the frequencies of high producers individuals for cytokines from the Th1 and Th2 profiles, like IL-4, IL-13, and IFN-γ. Multivariate analyses can facilitate understanding how enzyme expression in *S. mansoni* eggs influences the host immune response since it allows the grouping of all important variables.

We hypothesize that the action of SmATPDase 1 and SmATPDase 2 individually generates a smaller amount of the AMP substrate that will be converted into adenosine through the action of the SmAP, SmNPP-5, and ecto-5'-nucleotidase enzymes. This smaller amount of adenosine generated will act preferentially on pro-inflammatory receptors (A1 and A3) on the surface of immune cells, leading to the assembly of a mixed response profile characterized by the presence of Th1 (IFN-gamma, TNF, IL-6, and IL-2) and Th2 (IL-4 and IL13) cytokines. On the other hand, both enzymes on the surface of the parasite's egg increase the amount of AMP that can be converted to a more significant amount of adenosine. This molecule will act preferentially on immune cell inhibition receptors (A2A and A2B), leading to an increase in the regulatory cytokine IL-10 and, consequently, modulating the immune response in the infected individual (Fig. 8) [74, 77].

It is important to emphasize that further studies are still needed to validate these findings since the present study has limitations regarding the number of samples evaluated. Another limitation is the use of fecal samples that may contain organic and inorganic substances with inhibitory effects on PCR. In addition, other schistosome proteins can regulate IL-10 function. However, our data support the relevance of studies in human beings investigating elements of the parasite and the cell-mediated immune response as potential candidates for future therapeutic interventions against schistosomiasis. Thus, the SmATPDases may be potential candidates for future therapeutic interventions against schistosomiasis. Other authors have already demonstrated the regulatory function of the IL-10 cytokine [55,73,78–80]; However, our study expands on this concept since it provides evidence that the enzymes SmATPDase 1 and SmATPDase 2 are important factors for maintaining IL-10 levels in the presence of *S. mansoni* eggs.
Conclusions

The expression of SmATPDases in *S. mansoni* eggs seems to influence the immune response of infected individuals, where individuals with the expression of both enzymes in fecal samples negatively modulate the host immune response most likely mediated by IL-10.

Abbreviations

SmATPDase 1: *Schistosoma mansoni* NTPDase 1; SmATPDase 2: *Schistosoma mansoni* NTPDase 2; eIF4E: eukaryotic translation initiation factor 4E; IL-10: interleukin-10; IFN-γ: interferon-γ; TNF: alpha-tumor necrosis factor; IL-4: interleukin-4; IL-6: interleukin-6; IL-2: interleukin-2; IL-13: interleukin-13; Th1: T helper 1; Th2: T helper 2; PBMC: Peripheral blood mononuclear cells; SmAP: schistosome alkaline phosphatase; SmPDE: schistosome cyclic nucleotide phosphodiesterases; SmNPP-5: schistosome tegumental phosphodiesterase 5; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; cAMP: cyclic adenosine monophosphate; qPCR: quantitative polymerase chain reaction; RTPCR: Reverse transcription polymerase chain reaction; RNA: ribonucleic acid; cDNA: complementary deoxyribonucleic acid; SYBR Green: N’, N’-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine; SEA: *Schistosoma mansoni* soluble egg antigen; MCA: Multiple Correspondence Analysis; epg: eggs per gram; HP (high producer).

Declarations

Acknowledgments

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-MCTI 14/2014 #454136/2014-5). The authors thank the Program for Technological Development in Tools for Health-RPT-FIOCRUZ for using the flow cytometry facilities. LCCA and ATC received PQ fellowships from CNPq.

Funding:

PML - #454136/2014-5, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-MCTI 14/2014). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information file.

Author’s contributions

Conceptualization: Pauline Martins Leite, Luís Carlos Crocco Afonso, Andréa Teixeira-Carvalho
Data curation: Pauline Martins Leite, Thalisson Artur Ribeiro Gomides.

Formal analysis: Thalisson Artur Ribeiro Gomides, Pauline Martins Leite, Márcio Luís Moreira de Souza.


Software: Thalisson Artur Ribeiro Gomides, Márcio Luís Moreira de Souza.

Supervision: Luís Carlos Crocco Afonso, Andréa Teixeira-Carvalho, Pauline Martins Leite.

Validation: Márcio Luís Moreira de Souza, Luís Carlos Crocco Afonso, Andréa Teixeira-Carvalho, Pauline Martins Leite.

Visualization: Thalisson Artur Ribeiro Gomides, Pauline Martins Leite.

Writing – original draft: Thalisson Artur Ribeiro Gomides, Luís Carlos Crocco Afonso, Pauline Martins Leite.

Writing – review & editing: Thalisson Artur Ribeiro Gomides, Márcio Luís Moreira de Souza, Luís Carlos Crocco Afonso, Andréa Teixeira-Carvalho, Pauline Martins Leite.

Ethics approval and consent to participate

This study was approved by the Ethics Committee at the Federal University of Juiz de Fora and is registered at the National Brazilian Platform for Research with Human Subjects under the following number: CAAE #44225715.6.0000.5147. All subjects gave written and signed informed consent. In the case of minors, additional written informed permission was obtained from their parents or guardians.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

**Figure 1**

Flowchart illustrating procedures performed to classify the study population.
Figure 2

Stimulation with SEA increases the production of inflammatory cytokines TNF, IL-6, IFN-γ and IL-2 (a), Th2 cytokines IL-4, IL-13, and immunomodulatory cytokine IL-10 (b) in individuals infected by *S. mansoni*. Levels of cytokines were measured in supernatants collected from SEA-stimulated PBMC cultures by multiplex assay. Peripheral blood mononuclear cells (PBMC) were stimulated with SEA (SEA) or were not stimulated (CC), and the production of cytokines in the supernatant was compared. The Mann-Whitney test was used for the statistical evaluation. ***Values of p<0.001 were considered significant.

Figure 3
Overall cytokines signatures at the distinct expression of SmATPDAses. The cytokines signatures were built, taking the proportion of subjects above the cut-off edges defined for each cytokine, calculated as the median index value (SEA/CC) for the study population. Diagrams were constructed for all study groups to calculate the proportion (%) of individuals above the median cut-off index for each cytokine (black-shaded spots). In the last line of diagrams, the value of the frequencies (%) of high-producing individuals for each cytokine is observed. (a) The undetectable SmATPDase group (individuals who did not show expression of SmATPDases in their stool samples) was used to construct an ascendant reference curve, used for comparative analysis amongst the study groups, (b) SmATPDase 1 (who express only SmATPDase 1 in their feces samples), (c) SmATPDase 2 (individuals who express only SmATPDase 2 in their feces samples), (d) Both enzymes (individuals who express both enzymes in their feces samples) and (e) Uninfected Controls (individuals no infected of S. mansoni). Substantial changes in the relevant cytokines were highlighted by (*) when the proportion of subjects above the cut-off shifted from quartile to more or less. The graphs show dotted lines at frequencies of 25%, 50%, and 75%, thus delimiting four quartiles (0 to 25%, 26 to 50%, 51 to 75%, and 76 to 100%). The relevant cytokine in each study group was underscored.

Figure 4

Expression of both enzymes modulates the IFN-γ production by IL-10. The IFN-γ/IL-10 ratio (SEA) was compared between the following groups: individuals who express only SmATPDase 1, only SmATPDase 2,
and individuals who express both enzymes. The Mann-Whitney test was used for the statistical evaluation, and $p < 0.05$ were considered significant.

**Figure 5**

*Radar graph representing the balance of cytokines induced by expression of only SmATPDase 1, only SmATPDase 2, and both enzymes in *S. mansoni* eggs extracted from infected individuals.* The panoramic profile of cytokines produced by individuals who express SmATPDase 1, and SmATPDase 2, Both as Enzymes or do not express an enzyme (Undetectable SmATPDase) in parasite eggs, was constructed as described in Methods. Data are presented in radar charts as the proportion of individuals with cytokine index (Ag-SEA / Control) above the overall median values for all subjects involved in the study. Cytokines with an index above the global median in more than 75% of individuals were highlighted by asterisks (*). The Venn diagram shows the intersections of common attributes presented by the three groups studied: SmATPDase 1 (Blue), SmATPDase 2 (Pink), and Both enzymes (green). Venn diagram report summarizing selected attributes with patterns labeled as shared by the SmATPDase 1 and SmATPDase 2 expression, exclusive of the SmATPDase 1 expression, unique to the SmATPDase 2 expression, and exclusive to the group that expresses both enzymes (intersection table).
Figure 6

Expression of SmATPDases contributes to the positive association between infection intensity and IL-10 index. Correlation between IL-10 index and *S. mansoni* parasitic load (EPG) was evaluated in the following groups: All *S. mansoni* infected population in the study. Individuals that did not present *S. mansoni* eggs with SmATPDase expression - Undetectable SmATPDase (gray). Individuals who had eggs and expressed at least one of the SmATPDases - Positive SmATPDase. The individuals were segregated according to the expression of SmATPDases: SmATDase 1 (orange), SmATPDase 2 (green), and both enzymes (blue). p <0.05 indicates statistically significant differences (Spearman correlation).
Figure 7

**Multiple correspondence analyses applied to the variables addressed in the study.** Multiple Correspondence Analysis (MCA) has been used to identify two principal components, which explain 37.34% of the variation in the dataset. In (a), the categories of analyzed variables are represented; (1) first quadrant; (2) second quadrant; (3) third quadrant; (4) fourth quadrant, (b) the contribution of variables about dimension one and dimension 2. In (c), the distribution of the *S. mansoni* infected individuals is observed, considering SmATPDases expression: SmATPDase 1 (orange); SmATPDase 2 (green); both enzymes (blue); undetectable expression of the enzymes (black). In (d), individuals expressing at least one of the enzymes were emphasized. The dotted lines highlight the presence of a cluster for enzymatic expression.
Figure 8

Schematic representation of the hypothetical molecular mechanism by which the enzymes SmATPDase 1 and SmATPDase 2 alter the host’s immune response. **A** and **b**) The expression of SmATPDase 1 or SmATPDase 2 alone in *S. mansoni* eggs leads to the production of adenosine in low concentrations, which can favor signaling via A1 and A3 receptors in the immune cells and, consequently, to the production of a mixed profile cytokines (Th1 and Th2). **c**) The expression of SmATPDase 1 and SmATPDase 2 concomitantly in *S. mansoni* eggs can cause the production of adenosine in high concentrations, favoring the production of a modulatory cytokine pattern (IL-10) by immune cells via A2A and A2B receptors.

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