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Schistosoma heamatobium tetraspanins TSP-2 and TSP-6 induce Dendritic Cells maturation, cytokine production and T helper cells differentiation *in vitro*

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ABSTRACT

Urogenital schistosomiasis caused by *Schistosoma haematobium* is a major cause of disability in endemic areas. Despite its socio-economic burden, no vaccine exists and the parasite's immunobiology remains underexplored. Genome annotation has revealed over 40 different genes encoding tetraspanins, transmembrane proteins with known immunomodulatory properties in other plathelminthes. This study investigated the role of *Sh*-TSP-2, *Sh*-TSP-6 and *Sh*-TSP-23, which are expressed in the parasite's tegument and extracellular vesicles (EVs). Immature dendritic cells (DCs) from unexposed healthy donors were stimulated with these proteins to evaluate maturation maker expression and cytokine production. Also, pre-activated T $CD4^+$ cells were stimulated with the DCs supernatant to assess cytokine gene expression. *Sh*-TSP-2 and *Sh*-TSP-6 induced maturation markers and cytokine production in DCs: *Sh*-TSP-2 increased CD80 and CD83 levels and the concentration of both pro-inflammatory (IL-6, TNF) and regulatory (IL-10) cytokines, while *Sh*-TSP-6 increased the production of IL-6. Moreover, supernatants from *Sh*-TSP-2 stimulated DCs induced the expression of Th1 (IFNɣ) and regulatory (IL-10) cytokines in $CD4^+$ T cells, while *Sh*-TSP-6 induced Th2 (IL-4, IL-13) cytokine expression. These results provide evidence that *S. haematobium* tetraspanins modulate the response of human DCs and $CD4^+$ T cells *in vitro,* and support *Sh*-TSP-2 as a promising vaccine candidate.

1. Introduction

Human schistosomiasis is a poverty-associated and neglected parasitic disease caused by trematode blood flukes of the genus *Schistosoma*. It is estimated that 250 million people are infected in 78 endemic countries, and that 90 % of cases occur in Sub-Saharan Africa (SSA) [1–3]. The immune response to the parasite is the main cause of pathology and clinical manifestations [4,5]. While acute schistosomiasis, with a non-specific presentation including fever, chills, rigors and myalgias, is due to reactions to larval stages, chronic schistosomiasis is due to response to the worm's eggs and causes most of the disease burden. *Schistosoma mansoni, S. intercalatum*, *S. guineensis*, *S. japonicum* and *S. mekongi* lay eggs in the mesenteric venous plexus and cause hepato-intestinal disease. *S. haematobium* is the only species laying eggs in the bladder venous plexus and in veins draining the genital tract and causing urogenital disease. *S. heamatobium* is endemic in Africa and the Middle East, and since recent years in some areas of Europe [6–8]. Clinical manifestations include haematuria, obstructive uropathy, kidney damage and squamous cell carcinoma. In females, genital schistosomiasis (FGS) causes ulcerative lesions and fibrosis in vagina, cervix

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and uterus, resulting in bleeding, pain, stigma, infertility and higher risk of HIV infection [9,10].

Despite the socio-economic and public health importance of schistosomiasis, no vaccines are commercialised yet [11]. Most of the efforts have been put in the development of a protective vaccine against *S. mansoni*, with several candidates (including Sm23, SmCB, SmG3PDH, Sm29 or paramyosin) providing promising results in pre-clinical animal model studies (reviewed in Ref. [12]) where a significant protection associated with Th1 and Th2 responses and a reduction in egg burden was shown. The Sm14 candidate (a fatty acid binding protein with a putative role in the transport of fatty acids from host cells) has reached phase 1b trials, showing good safety and immunogenicity and paving the way to phase 2 trials [13,14]. As for *S. haematobium*, the Sh28GST candidate (a glutathione-*s*-transferase involved in parasite metabolism) reached phase 3 trials, although a sufficient efficacy was not achieved probably due to several factors including interference by drug treatment and the chosen vaccine-administration regimen [15].

Another candidate currently undergoing phase 2 trials (NCT03910972) is *Sm*-TSP-2, a membrane protein belonging to the tetraspanin (TSP) family that has been identified in the tegument and extracellular vesicles (EVs) of *S. mansoni* [12,16,17]. TSPs play an important role in tegument formation, maturation and stability in schistosomes and other platyhelminths, and have been identified abundantly in their secretome, tegument and EVs [12,18–20]. Additionally, their diagnostic efficacy has been highlighted for the diagnosis of urogenital schistosomiasis and other helminthiases such as taeniasis [21,22]. TSPs consist of four transmembrane domains, a small extracellular loop (SEL) and a large extracellular loop (LEL) and are involved in many cellular activities such as cell proliferation, differentiation, adhesion, and division [23]. Between four and eight cysteine residues forming two to four disulfide bonds are present in the LEL region of TSPs, serving as a distinctive "signal motif' that allows for specific protein-protein interactions with neighbouring proteins and other ligands [24]. The immunisation of mice with the *Echinococcus granulosus* TSP-1 (*Eg*-TSP-1) induced the upregulation of splenic IL12, IL10 and IFNγ but not of IL4, suggesting its role in the modulation of the immune response towards a Th1 phenotype along with the activation of self-limiting regulatory responses [25]. Recently, the *Opisthorchis viverrini* TSPs *Ov*-TSP-2 and *Ov*-TSP-3 have been shown to promote IL6 and IL8 responses and to stimulate cell proliferation in human cholangiocytes, which might explain, in part, the carcinogenicity effect of this liver fluke [26].

Annotation of recently sequenced *S. haematobium* genomes has revealed over 40 different genes encoding for tetraspanins in this blood fluke. From these, at least 6 proteins have been identified in the tegument or EVs of *S. haematobium*, including *Sh*-TSP-2, *Sh*-TSP-4, *Sh*-TSP-5, *Sh*-TSP-6, *Sh*-TSP-18 and *Sh*-TSP-23 [20]. While *Sh*-TSP-2, *Sh*-TSP-6 and *Sh*-TSP-23 clustered together in the CD63 family and were expressed in all life stages and in particular in schistosomula and adult worms, *Sh*-TSP-4, *Sh*-TSP-5 and *Sh*-TSP-18 grouped under the uroplakin family of TSPs and were expressed mainly in egg, miracidia and cercariae [27]. The presence of these molecules on the tegument and/or internal organs of the parasite and their ability to be recognized by the host's immune system and to induce immune responses make them ideal candidates for the diagnosis and control of urogenital schistosomiasis [22]. Indeed, *Sh*-TSP-2, *Sh*-TSP-6 and *Sh*-TSP-23, as well as other CD63 members from different trematodes, have been selected as potential diagnostic markers, since they can be recognized by the sera of infected patients [22] and have been tested in vaccination studies [28]. However, despite the interest of these antigens for vaccine development, there is very little information about their role in the immunobiology of urogenital schistosomiasis.

The aim of the present study was therefore to assess the immunomodulatory role of *S. haematobium* TSP-2, TSP-6, TSP-23 by testing their ability to induce dendritic cells (DCs) maturation and cytokine production, as well as to affect T helper cell differentiation *in vitro*.

2. Materials and methods

2.1. Recombinant protein production and purification

The molecules *Sh*-TSP-2, *Sh*-TSP-6 and *Sh*-TSP-23 were selected from all tetraspanins identified in the *S. haematobium* proteome, as they are primarily expressed during the life stages occurring in the definitive host and also because they can be expressed in a soluble form, unlike the members of the uroplakin family. The extracellular loop 2 of these proteins were expressed and purified as described previously [27]. Briefly, *E. coli* BL21(DE3) containing plasmids encoding for these proteins were inoculated into 10 mL of Luria broth containing 100 μg/mL ampicillin (LB_{amp}; in the case of *Sh*-TSP-6 and *Sh*-TSP-23) or 50 μg/mL kanamycin (LB_{kan}; in the case of *Sh*-TSP-2) and incubated overnight at 37 °C with shaking at 200 rpm. Overnight culture was seeded (1/100) into 500 mL of fresh LB_{amp} and incubated at 37 °C with shaking at 200 rpm until $OD_{600} = 0.5–1.0$ (approximately 3 h), whereupon expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Bioline, UK) and cultured for 4 h (*Sh*-TSP-6 and *Sh*-TSP-23) or 16 h (*Sh*-TSP-2). Each pellet was resuspended in 50 mL of lysis buffer (50 mM sodium phosphate pH 8, 40 mM imidazole and 300 mM NaCl), and subjected to 3 cycles of freeze/thawing 3 ties sonication (5 s bursts x 10 times) at 4 °C. The bacterial lysate was finally centrifuged at 20,000 *g* for 20 min at 4 °C and the supernatant decanted and stored at −80 °C.

An AKTA Pure UPC FPLC (GE Healthcare, USA) was used to purify the recombinant proteins using Ni^{2+} immobilized metal ion affinity chromatography (IMAC). The recombinant protein solutions were appropriately diluted at a ratio of 1:4 in buffer A (50 mM sodium phosphate pH 8 and 300 mM NaCl) and subsequently passed through a 0.45 μm filter. These solutions were introduced into a 1 mL His-Trap IMAC column (GE Healthcare, USA) that had been pre-equilibrated with buffer A at a flow rate of 1 mL/min. Bound proteins were washed with 10 column volumes (CV) of buffer A and then eluted using buffer A with an increasing linear gradient of imidazole (100–500 mM). Fractions containing purified recombinant proteins with the highest purity were pooled and subjected to buffer exchange into PBS using an Amicon Ultra-15 centrifugal filter with a molecular weight cutoff (MWCO) of 3 kDa. Thioredoxin tags were cleaved from *Sh*-TSP-6 and *Sh*-TSP-23 using the enzyme entoerkinase. The step was not performed for *Sh*-TSP-2 since it was expressed without any tag. The identity of expressed proteins was confirmed by SDS-PAGE and Western blot using anti-His monoclonal antibodies.

Lipopolysaccharides (LPS) was removed from purified recombinant proteins using Lionex Endotrap HD columns (Lionex, Braunschweig, Germany) as per manufacturer's instructions. Proteins were quantified using Bradford assay with a BSA standard curve.

Endotoxin contamination in recombinant TSPs was evaluated by the ToxinSensor Chromogenic LAL Endotoxin assay kit (GenScript, New Jersey, USA). Briefly, 100 μL of tetraspanins at the concentration of 10 μg/ml (corresponding to 1 μg of recombinant protein) were mixed with the LAL reagent in endotoxin-free vials. 200 μl of the final solution were transferred into 96-well plate and the absorbance was measured at 545 nm using Victor Nivo (PerkiElmer, USA). Residual contamination of LPS was revealed at a concentration of 0.105 EU/ml/, 0.01 EU/ml, 0.05 EU/ml in *Sh*-TSP-2, *Sh*-TSP-6, *Sh*-TSP-23 respectively. The same results expressed in micrograms, show an LPS concentration of 0.105 EU/μg, 0.01 EU/μg, 0.05 EU/μg, in *Sh*-TSP-2, *Sh*-TSP-6, *Sh*-TSP-23 respectively. Since LPS concentration in *Sh*-TSP-2 exceeds the limit of 0.05 EU/μg suggested for cell cultures [29], DCs experiments with *Sh*-TSP-2 were repeated in presence of colistin, an LPS inhibitor [30].

2.2. Ethical approval

The use of buffy coats from donated blood, not useable for therapeutic purposes, was approved by the Ethics Committee of the Azienda

Ospedaliera Universitaria Careggi (AOUC, Firenze, Italy) in agreement with the D.M. of the Italian Ministry of Health (15A09709) G.U., n. 300 12.28. 2015).

2.3. Cells isolations and culture conditions

Mononuclear Cells were isolated by Ficoll/Paque (GE Healthcare, Italy) density gradient centrifugation from buffy coats obtained from five healthy donors. $CD14^+$ cells were separated performing a positive selection using mouse anti-human CD14 conjugated magnetic microbeads (Miltenyi Biotec, Germany) by magnetic cell sorting (MACS), according to the manufacturer's instructions. The cells were seeded at 1×10^6 cells/mL. Monocyte-derived DCs were generated by culture of $CD14^+$ in RPMI 1640 with 10 % heat-inactivated foetal bovine serum (FBS), 100U/mL penicillin and 0.1 ng/mL streptomycin with 1000 U/mL rGM-CSF and 1000 U/mL IL-4 for 7 days, at 37 °C in a humidified chamber with 5 % CO_2 [31].

$CD4^+$ T cells were isolated from non-adherent fraction of PBMCs of three donors or from buffy coats obtained from two healthy donors using a $CD4^+$ T cells separation Kit (Miltenyi Biotec) and were seeded in RPMI 1640 with 10 % heat-inactivated FBS, 100U/mL penicillin and 0.1 ng/mL streptomycin at 37 °C in a humidified chamber with 5 % CO2.

2.4. DCs maturation

Maturation of DCs was induced in RPMI 1640 with 10 % heat-inactivated foetal bovine serum (FBS), 100U/mL penicillin and 0.1 ng/mL streptomycin and either 0.1 μg/mL LPS or 10 μg/mL *Sh*-TSP-2 or 10 μg/mL of *Sh*-TSP-6 or 10 μg/mL *Sh*-TSP-23 for 16 h. Unstimulated DCs were used as the maturation negative control and LPS as maturation positive control.

2.5. Flow cytometry

Following DCs maturation, cells were washed and stained with a mixture of anti-CD83-PE, anti-CD80-FITC, anti-CD86-APC and anti-HLA-DR-APC antibodies (BD Biosciences-Pharmingen) for 30 min. Cells were analysed using the ACCURI instrument (BD Biosciences, NJ, USA) using Cflow Plus software (BD Biosciences, NJ, USA). Ten thousand events for each sample were acquired [31]. Results were expressed as Mean Fluorescence Intensity (MFI; Supplementary methods). The experiment was conducted in three different donors (biological replicas).

2.6. Luminex assay

After the 16 h of DCs maturation, supernatants were collected and cytokines levels (IL1β, IL6, TNF, IL12p70, IL23, IL4, IL13, IL10) estimated using the Milliplex Human Th17 Magnetic Bead Panel for Luminex MAGPIX detection system (Affymetrix, eBioscience) following the manufacturer's instructions. Results were expressed as concentration (pg/ml). The experiment was conducted in five different donors (biological replicas).

2.7. Dose-response and colistin assays

DCs maturation was induced in RPMI 1640 with 10 % heat-inactivated foetal bovine serum (FBS), 100U/mL penicillin and 0.1 ng/mL streptomycin, and either 0.1 μg/mL LPS or 1 μg/mL *Sh*-TSP-2 or 10 μg/mL of *Sh*-TSP-2 or 100 μg/mL of *Sh*-TSP-2 for 16 h, in absence and presence of 10 μg/mL colistin (Sigma Chemical Co. St. Louis, MO). Unstimulated DCs were used as the maturation negative control and LPS as maturation positive control. Maturation markers and cytokine concentration were assessed by Flow Cytometry and Luminex assay as above. The experiment was conducted in three different donors (biological replicas).

2.8. T cells differentiation

Isolated CD4+T cells (8 or $2x10^5$ cells/mL) from five healthy donors were activated by anti-CD3/CD28 antibodies coupled to beads at a 1:1 bead/cell ratio (Dynabeads™ Human T-Activator CD3/CD28, Gibco, Thermo Fisher Scientific, MA, USA) and incubated with conditioned medium obtained from cultures of DCs with LPS or tetraspanins (*Sh*-TSP-2, *Sh*-TSP-6, and *Sh*-TSP-23) for 3 days.

2.9. RNA extraction

T cells RNA extraction was performed using 1 mL of TRIzol® (Invitrogen) reagent. 200 μL of chloroform per mL of TRIzol® were added and samples were centrifuged at 12000 g for 15 min at 4 °C to separate the homogenate into three phases: a clear upper aqueous layer containing RNA, an interphase and a red lower organic layer containing DNA and proteins. The RNA phase was collected, resuspended in 500 μL of isopropanol per mL TRIzol® and precipitated by centrifuging samples at 12000 g for 10 min at 4 °C. Afterwards, the pellet was washed with 1 mL of 75 % and then with 1 mL of 100 % ethanol. When the ethanol was evaporated (30′ minutes) the pellet was resuspended in H2O RNase-free.

2.10. RNA quantification

RNA amount was assessed by the NanoDrop spectrophotometer (ThermoFisher Scientific, Waltman, MA, USA), using 2 μL of sample according to the instrument software's instructions and stored at −80 °C.

2.11. Retro transcriptase quantitative PCR

A quantity of 1 μg of RNA was used for the reverse transcription (RT) reaction with Prime Script RT reagent Kit Takara (Otsu, Japan). After treatment with DNase for 30 min at room temperature, the cDNA samples obtained were amplified using specific primers (Supplementary Table 1) to quantify IFNg, IL17, IL4, IL5, IL13 and IL10 mRNA expression. RTqPCR amplification was carried out using SYBR Premix Ex Taq (Takara) according to manufacturer instructions on a Rotorgene RG-3000 A cycle system (Qiagen) platform. The 18S rRNA housekeeping gene was used as a normalizer [32], and the difference between CT values of the target gene and 18S gene was used to calculate the delta CT. Cytokine gene expression level was expressed as relative amount using the $2^{-\Delta Ct}$ method. The experiment was conducted in three different donors (biological replicas).

2.12. Statistical analysis

Difference in the distribution of quantitative results (i.e., DCs maturation marker MFI, DCs cytokine concentration, T helper cells cytokine gene expression level) between stimulated and unstimulated cells was assessed using Kruskal-Wallis non-parametric rank test. The fold-change in the value of interest observed in stimulated compared to unstimulated cells (US) was computed for each donor, and the distribution among donors was shown using boxplots. The analysis was performed in GraphPad Prism v.9.0.

3. Results

All TSPs were expressed successfully in a soluble form using a bacterial expression system. Since TSPs are transmembrane proteins, only the extracellular loop (EC) 2 from these proteins was expressed. This loop was also selected because of its antigenicity, since intracellular parts and short EC loop 1 are not involved in protein binding (Hemler, 2008). DCs obtained from five different donors were cultured for 16 h in absence (negative control) or presence of 10 μg/mL of tetraspanins (*Sh*-TSP-2, *Sh*-TSP-6, *Sh*-TSP-23) or 0.1 ng/mL LPS (positive control), and

the expression of DCs maturation markers HLA-DR, CD80, CD83 and CD86 was measured by cytofluorimetric analysis. As shown in Fig. 1 and Supplementary Table 2, *Sh*-TSP-2 induced the expression of CD80 (3-fold change) and CD83 (7-fold change) maturation markers, with a significant increase compared to unstimulated cells. A similar increase in CD80 (2-fold change) and CD83 (3-fold change) expression was induced by *Sh*-TSP-6, although the comparison to unstimulated cells was only close to significance (P-value≤0.10). No increase in the expression of maturation markers was observed upon *Sh*-TSP-23 stimulation.

Conditioned media from DCs cultured as reported above were used to assess the production of cytokines involved in T cell activation and effector differentiation by Immunoplex assay. As shown in Fig. 2 and Supplementary Table 3, *Sh*-TSP-2 strongly induced the production of inflammatory cytokines IL6 (478-fold change) and TNF (219-fold change), and, to a lesser extent, IL1β (6-fold change), with a significant increase compared to unstimulated cells for the three cytokines. A similar although less pronounced effect was observed for *Sh*-TSP-6, which significantly induced the production of IL6 (121-fold change), whereas the induction of TNF (7-fold change) and IL1β (3-fold change) was only close to significance (P.value ≤ 0.10) compared to unstimulated cells. On the other hand, there was no evidence that T *Sh*-TSP-23 induced the production of neither IL1β, IL6 or TNF. *Sh*-TSP-2 significantly induced the production of the Th1 cytokine IL12p70 (7-fold change), whereas no differences compared to unstimulated cells were observed for *Sh*-TSP-6 and *Sh*-TSP-23. Similarly, *Sh*-TSP-2 induced the production of the Th17 cytokine IL23 (5-fold change), while no effect was observed for *Sh*-TSP-6 and *Sh*-TSP-23. *Sh*-TSP-2 had a negligible although significant effect on the production of Th2 cytokine IL13 (1.6-fold change) but not of IL4. No effect on the production of Th2 cytokines was observed for *Sh*-TSP-6 and *Sh*-TSP-23. Finally, *Sh*-TSP-2 strongly and significantly induced the production of regulatory cytokine IL10 (342-fold change). A much milder effect was observed for *Sh*-TSP-6 (12-fold change) which was only close to significance ($p < 0.1$), while no effect was observed for *Sh*-TSP-23. In summary, stimulation of DCs with *Sh*-TSP-2 resulted in a large increase in the production of inflammatory cytokines IL6 and TNF as well as of regulatory IL10 cytokine, and at the same time a modest increase in inflammatory and Th1/Th17 cytokines IL1β, IL12p70, IL23. Stimulation with *Sh*-TSP-6 had similar although more modest effects, while stimulation with *Sh*-TSP-23 had no effect of cytokine production by DCs.

The DCs response to *Sh*-TSP-2 was further investigated by evaluating the antigen dose-response effect as well the possible interference of any residual endotoxin contamination in the protein preparation on DCs maturation and cytokine production. To this end, cultures were incubated with either *Sh*-TSP-2 at three different concentrations (1 μg/mL, 10μ/mL or 100 μg/mL), both in presence and in absence of colistin, an LPS inhibitor, and the expression of maturation marker CD83 as well as cytokines concentration in the conditioned media were assessed. Regarding the expression of CD83, while colistin reduced the response to LPS as expected, it did not reduce the response to *Sh*-TSP-2. Also, the

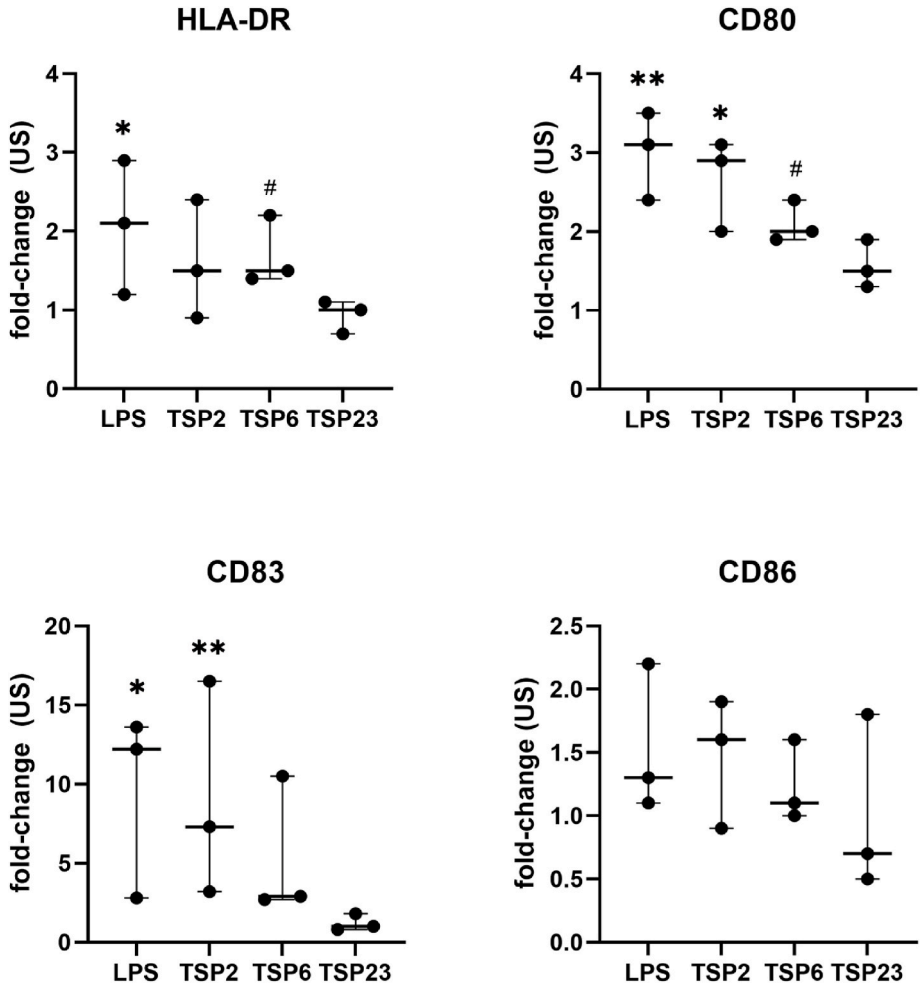

Fig. 1. DCs expression of maturation markers upon stimulation with *S. haematobium* TSP-2, TSP-6 and TSP-23.
The figure shows individual datapoints and boxplots (median and 5–95 % range) of the fold-change in MFI of stimulated DCs (LPS, *Sh*-TSP-2, *Sh*-TSP-6, *Sh*-TSP-23) compared to unstimulated DCs (US). Asterisks indicated statistical significance of Kruskal Wallis rank test (#p value ≤ 0.10, * p-value≤0.05, ** p-value≤0.01, *** p-value≤0.001).

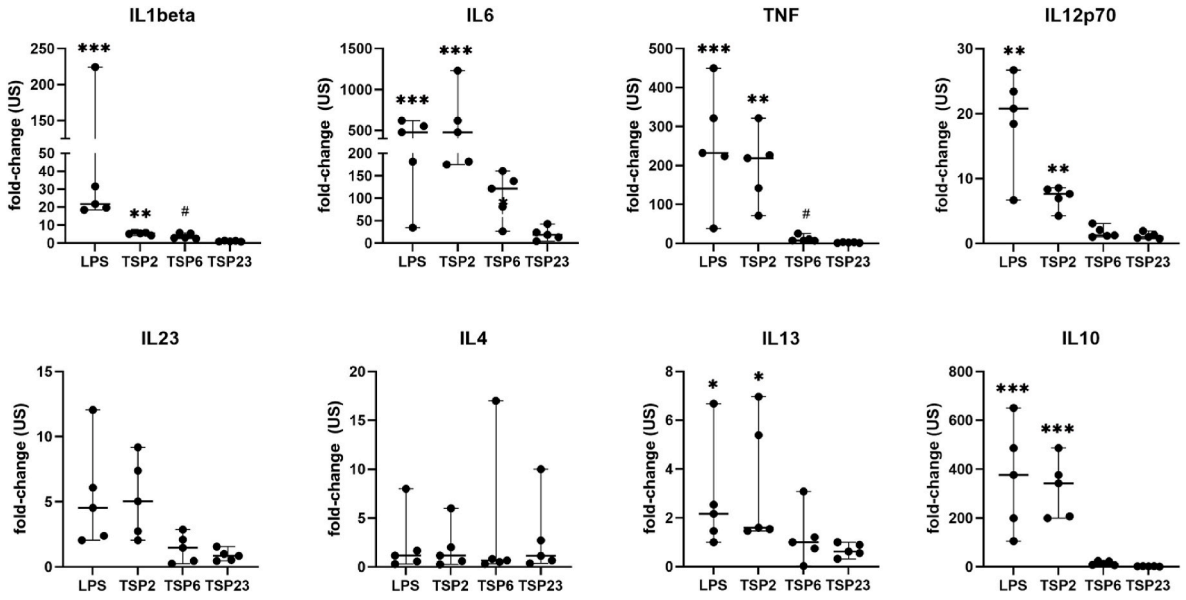

Fig. 2. DCs cytokine production upon stimulation with *S. haematobium* TSP-2, TSP-6 and TSP-23.
The figure shows individual datapoints and boxplots (median and 5–95 % range) of the fold-change in concentration of cytokines present in stimulated DCs supernatant (LPS, *Sh*-TSP-2, *Sh*-TSP-6, *Sh*-TSP-23) compared to those present in unstimulated DCs supernatant (US). Asterisks indicated statistical significance of Kruskal Wallis non parametric rank test (#p value ≤ 0.10, * p-value≤0.05, ** p-value≤0.01, *** p-value≤0.001).

fold-change in expression of this maturation marker was not significant when *Sh*-TSP-2 stimulus was provided at 1 μg/mL, increased to significance level at 10 μg/mL concentration, while no further increase was observed at 100 μg/mL concentration, indicating that 10 μg/mL concentration is optimal for stimulation assays with this cytokine (Fig. 3, top panel). Regarding cytokine production, similar results were observed. As an example, while colistin reduced the supernatant concentration of the regulatory cytokine IL10 following LPS stimulation, such reduction was not observed following *Sh*-TSP-2 stimulation. The fold-change in IL10 concentration was significant when *Sh*-TSP-2 was

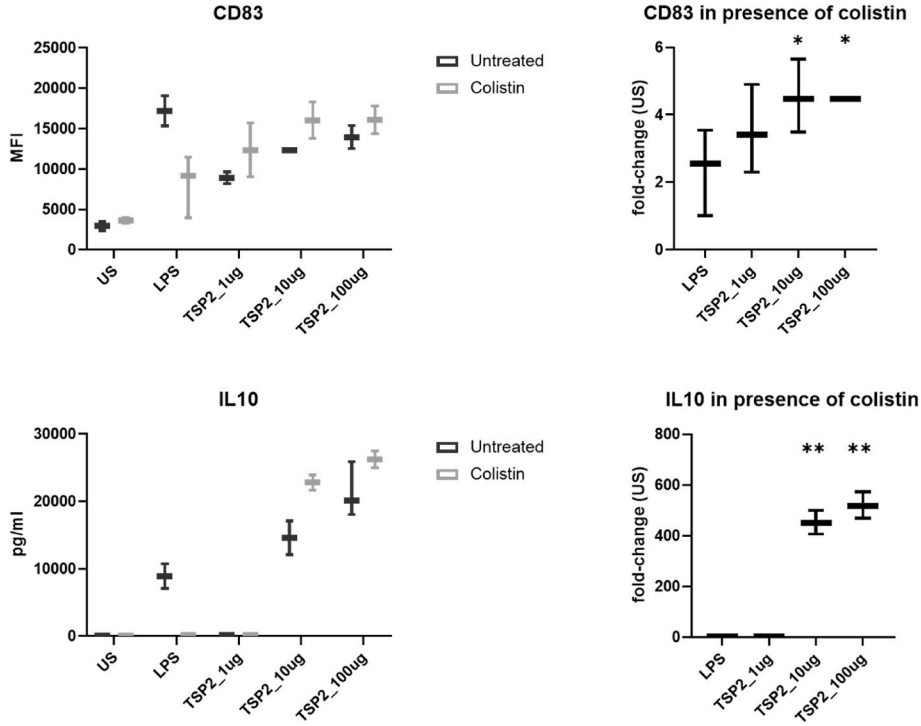

Fig. 3. DCs expression of CD83 and production of IL10 upon stimulation with *Sh*-TSP-2, in absence and presence of an LPS inhibitor.
The left panel include graphs showing boxplots (median and 5–95 % range) of CD83 expression (MFI, Mean Fluorescence Intensity; top) and IL10 production (pg/ml; bottom) by unstimulated DCs (US) and by DCs stimulated with LPS and *Sh*-TSP-2 and different concentrations (1 μg/mL, 10 μg/mL, 100 μg/mL) in absence (Untreated) and presence (Colistin) of the LPS inhibitor colistin. The right panel include boxplots (median and 5–95 % range) of the fold-change in level of CD83 expression (top) and IL10 production (bottom) by stimulated vs unstimulated (US) DCs. Asterisks indicated statistical significance of Kruskal Wallis rank test (* p-value≤0.05, ** p-value≤0.01, *** p-value≤0.001). The experiment was conducted with DCs from 3 donors.

used at a 10 μg/mL with no further increase at higher concentration (Fig. 3, bottom panel). Results for other cytokines are available as Supplemental Information.

Finally, in order to evaluate the ability of tetraspanins to affect T helper cell differentiation, conditioned media from DCs cultures were used to stimulate pre-activated T CD4$^+$ cells where cytokine gene expression levels were assessed by RTqPCR. As shown in Fig. 4 and Supplementary Table 4, the supernatants of tetraspanin-stimulated DCs were able to induce changes in cytokine gene expression in T CD4$^+$ T cells, with different pattern depending on the tetraspanin. *Sh*-TSP-2 was able to induce a significant increase in Th1 cytokine IFNɣ (3-fold change) and regulatory cytokine IL10 (2-fold change) gene expression but not in Th2 cytokines gene expression. *Sh*-TSP-6 was able to induce an increase in gene expression of Th2 cytokines IL4 (2-fold change) and IL13 (3-fold change), but not of Th1 or regulatory cytokines gene expression. To a smaller extent also *Sh*-TSP-23 was able to induce an increase in gene expression of Th2 cytokines IL4 (3-fold change) and IL5 (2-fold change), but only with close to significant differences compared to unstimulated cultures (P-value ≤0.10). No significant results were observed regarding IL17 gene expression, regardless of the tetraspanin evaluated.

4. Discussion

The data herein reported show that *Sh*-TSP-2 is able to induce the expression of maturation markers CD80 and CD83 and the production of inflammatory cytokine IL6 and TNF and regulatory cytokine IL10 in stimulated DCs *in vitro*. Furthermore, the supernatant from *Sh*-TSP-2 stimulated DCs is able to induce gene expression of Th1 cytokine IFNɣ and of regulatory cytokine IL10 in T CD4$^+$ cells *in vitro*. These effects are independent of any residual bacterial endotoxin contamination in the protein preparation as demonstrated by experiments conducted in the presence of an LPS inhibitor. As for *Sh*-TSP-6, the results show that this protein is able to induce the production of inflammatory cytokine IL6 in DCs, and gene expression of Th2 cytokines IL4 and IL13 in T CD4$^+$ cells. Finally, no evidence was obtained that *Sh*-TSP-23 has an immunomodulatory effect neither in DCs or CD4$^+$ T cells.

These results are worthy of confirmation in studies employing a larger number of donors and complementary methodologies. Also, further studies are envisaged that could identify the molecular targets of tetraspanins and their mode of action. Furthermore, since recombinant proteins expressed in prokaryotic expression systems are not glycosylated, and considering the immunomodulatory properties of glycans, expressing these tetraspanins in a eukaryotic system could shed light on the role of glycans in the responses found herein. Finally, the possibility that tetraspanins might be able to induce the differentiation of pre-activated T cells in absence of DC supernatants has not been ruled out, and could be investigated by future studies. However preliminary, the findings herein presented suggest an immunomodulatory role for *Sh*-TSP-2, which would be able to induce both inflammatory and regulatory responses in mature DCs and induce the differentiation of T CD4$^+$ cells towards Th1 and Tregulatory effector lymphocytes, or Th1 lymphocytes producing IL-10 to prevent excessive inflammation [33]. An increase in the expression of both Th1 (IFNγ) and regulatory (IL10) cytokines had been previously reported in mice following stimulation with *Eg*-TSP-1 [25]. An immunomodulatory role is also suggested for *Sh*-TSP-6 which would instead induce an inflammatory response in mature DCs and induce the differentiation of T CD4$^+$ cells towards the Th2 subpopulation. Such observations illustrate the complexity of the human immune response to the plethora of antigens presented by *Schistosoma* parasites.

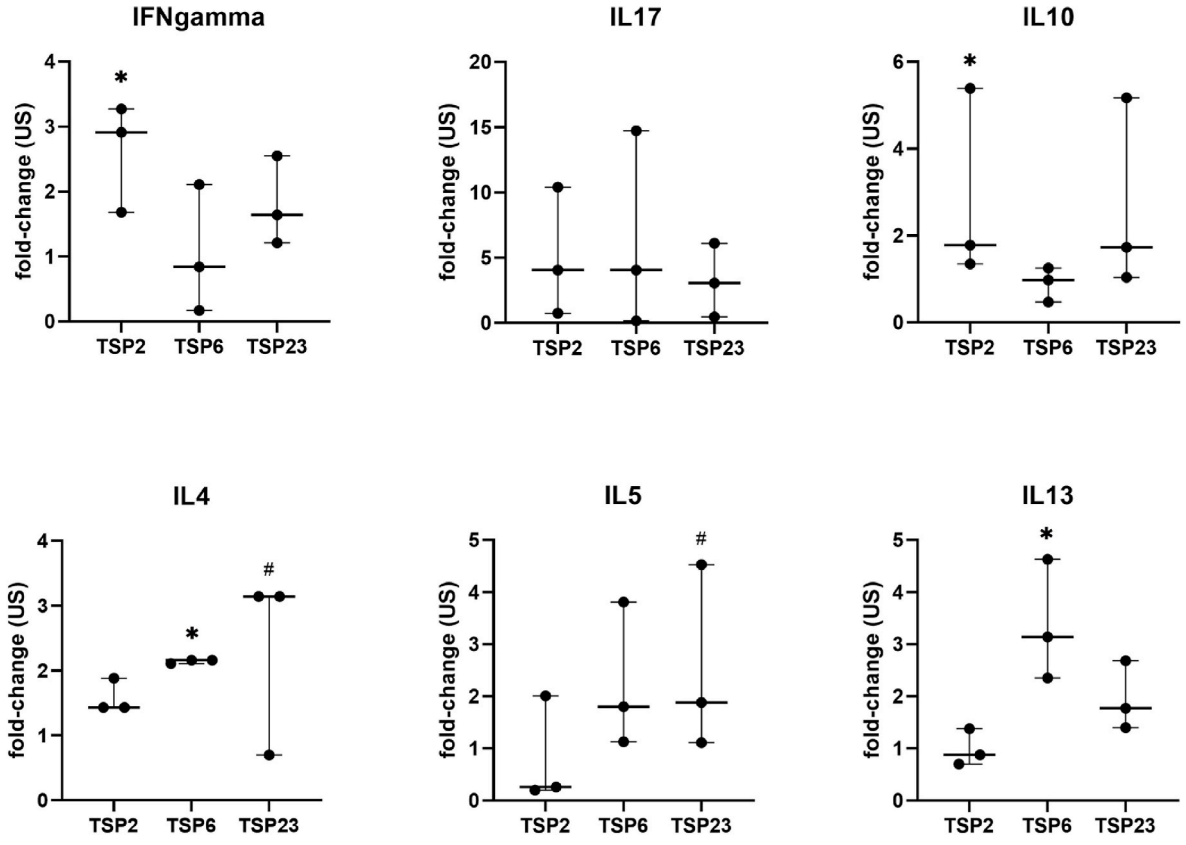

Fig. 4. T CD4$^+$ cells cytokine gene expression.
The figure shows individual datapoints and boxplots (median and 5–95 % range) of the fold-change in transcript relative amounts of T CD4$^+$ cells co-cultured with tetraspanin-stimulated DCs supernatant (*Sh*-TSP-2, *Sh*-TSP-6, *Sh*-TSP-23) compared to unstimulated T CD4$^+$ cells (US). Asterisks indicated statistical significance of Kruskal Wallis rank test (#p value ≤ 0.10, * p-value≤0.05, ** p-value≤0.01, *** p-value≤0.001).

Furthermore, they provide evidence that supports the choice of *Sh*-TSP-2 as a vaccine candidate against urogenital schistosomiasis. Indeed, *Sh*-TSP-2 is the homolog of the *Sm*-TSP-2 protein of *S. mansoni*, with which it shows 69.6 % sequence identity [20], a vaccine candidate showing promising results in phase 1b clinical trials [17] and currently undergoing phase 2 trials. Furthermore, vaccination with *Sh*-TSP-2 against heterologous challenge with *S. mansoni* has resulted in significantly lower tissue egg burden [28].

Tetraspanins are commonly found in EVs, and play crucial roles in their biogenesis, targeting, and function. For instance, *Sh*-TSP-2 has been identified in EVs from *S. haematobium* adult worms [20,28], although it has also been detected in the tegument of these worms, therefore some authors have suggested that EVs might not be the sole target of the immune response in vaccination studies [34]. Furthermore, the immunomodulatory effects of helminth EVs on both immune and non-immune cells have been extensively documented [35], with some experiments even demonstrating their potential as vaccine candidates [28,36]. However, our understanding on the specific bioactive molecules present in EVs remains limited. It has been recently shown that two different tetraspanins (*Ov*-TSP-2 and *Ov*-TSP-3) present in the EVs from the carcinogenic liver fluke *O. viverrini* can induce IL6 and IL8 responses and stimulate cell proliferation in human cholangiocytes [26]. This underscores the immunomodulatory potential of tetraspanin proteins and might explain, at least in part, the observed immunomodulatory effects of helminth EVs. Furthermore, *Schistosoma japonicum* EVs have been shown to drive macrophages differentiation towards an M1 phenotype, able to kill schistosomula by producing nitric oxide and to prevent liver fibrosis, and promote TNF production [37,38]. While some studies have implicated certain miRNAs in these responses [38], the significance of *Sh*-TSP-2 and other tetraspanins should not be overlooked in future investigations into trematode EVs.

As mentioned above, immunization with *Sh*-TSP-2, abundant both in EVs and tegument of *S. haematobium*, has been associated with a decrease in liver and intestine tissue egg burden of *S. mansoni* (heterologous challenge), which is an important hallmark in vaccine development against schistosomiasis as eggs play a major part in disease pathogenesis [28]. The results herein presented concerning *Sh*-TSP-2 contribute to the understanding of the mechanisms by which this protein could play a relevant role in parasite-host relationship and represent an optimal candidate for vaccine developments. Indeed, evidence has been gained that *Sh*-TSP-2 not only is immunogenic but might induce Th1 and T regulatory cells differentiation, suggesting that protective cellular [37,38] and cytophilic antibody [25,39] responses targeting the tegument of schistosomula could be elicited at the same time suppressing immunopathogenic responses. The combination of vaccination with mass drug administration with praziquantel represents the best strategy available to reach the control of schistosomiasis along the Neglected Tropical Diseases roadmap 2021–2030 [40].

CRediT authorship contribution statement

Angela Silvano: Writing – review & editing, Methodology, Investigation. **Javier Sotillo:** Writing – review & editing, Methodology, Investigation. **Marta Cecchi:** Writing – review & editing, Methodology, Investigation. **Alex Loukas:** Methodology. **Mireille Ouedraogo:** Writing – review & editing, Investigation. **Astrid Parenti:** Writing – review & editing, Methodology, Conceptualization. **Fabrizio Bruschi:** Writing – review & editing, Resources, Conceptualization. **Maria Gabriella Torcia:** Writing – review & editing, Resources, Formal analysis, Conceptualization. **Valentina D Mangano:** Writing – original draft, Resources, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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