Treatment with egg antigens of Schistosoma mansoni ameliorates experimental colitis in mice through a colonic T-celldependent mechanism

Treatment with egg antigens of *Schistosoma mansoni* ameliorates experimental colitis in mice via a colonic T cell-dependent mechanism.

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Conflicts of Interest: none

ABSTRACT

Background: Helminth-derived molecules are being identified as a new therapeutic approach for immune-mediated diseases. We investigated the anti-inflammatory effect and the immunological mechanisms of *Schistosoma mansoni* soluble egg antigens (*Sm*SEA) in a mouse model of chronic colitis.

Methods: Colitis was induced in immunocompromised SCID mice by the adoptive transfer of $CD4^+CD25^-$ $CD62L^+$ T cells. Two weeks post-transfer, *Sm*SEA treatments were started (study 1: 1x20µg *Sm*SEA/week/x5; study 2: 2x20µg *Sm*SEA/week/x3). From the start of the treatment (week 2), the clinical outcome and colonic inflammation were assessed at different timepoints by a clinical disease score and colonoscopy, respectively. At the end of the studies, the colons were harvested for macroscopic examination and colonic lamina propria mononuclear cells (LPMC) were isolated for flow cytometric T cell characterization.

Results: In both studies, administration of *Sm*SEA in colitis mice improved all the inflammatory parameters studied. However in study 1, this beneficial effect on inflammation diminished with time and the T cell characterization of the LPMC, performed at week 6, revealed no immunological effects of the *Sm*SEA treatment. In study 2, mice were sacrificed earlier (week 4) and at that timepoint, we found a significant downregulation of the number of IL-17A-producing T cells and a significant upregulation of the number of IL-4-producing T cells in the colon of the *Sm*SEA-treated colitis mice.

Conclusions: Our results demonstrate that the administration of *Sm*SEA reduces the severity of colitis in the adoptive transfer mouse model characterized by an increased Th2 response and a suppressed Th17 response in the colon.

Keywords: therapy with helminths, helminth-derived products, *Schistosoma mansoni* soluble egg antigens, colitis, inflammatory bowel disease

INTRODUCTION

Helminthic therapy as a possible approach to treat inflammatory bowel diseases (IBD) is being investigated extensively in animal models of IBD and in clinical trials.[1] It has been hypothesized that helminths can skew the disturbed intestinal immune balance in IBD patients towards a more immunosuppressive state through the induction of T helper (Th) 2 and regulatory T cell (Treg) immune responses, suppressing proinflammatory Th1 and Th17 responses and thereby suppressing intestinal inflammation.[2, 3] However, the negative results of two recent multicenter randomized double-blind placebo-controlled phase II trials conducted in the USA (TRUST-1, NCT01576471) and Europa (TRUST-2, NCT01279577) testing the efficacy of a 12 week treatment with *Trichuris suis* ova (TSO) in patients with moderate to severe Crohn's disease dampened the enthusiasm for TSO treatment and thus for helminth infection per se. Therefore an increasing interest in the identification and characterization of helminth-derived molecules responsible for the immunomodulatory anti-inflammatory effects of helminths as new therapeutic strategies for IBD emerged. McSorley *et al.* recently reviewed a whole range of helminth-derived mediators and their associated immunomodulatory mechanisms.[1, 4]

Schistosoma mansoni is identified as a potent natural inducer of Th2 responses. However, the development of this Th2 polarization only coincides with the onset of egg production by adult worms.[5, 6] In addition to Th2 responses, the importance of Treg during the egg deposition phase of *S. mansoni* infection has also been shown.[7] *S. mansoni* eggs thus contain or secrete molecules that trigger potent Th2 and Treg responses. Consequently, different products of *S. mansoni* eggs (e.g. omega-1, IPSE/alpha-1, lacto-N-fucopentaose III) have already been tested for their immunological effects.[5, 8-13] However, Bodammer *et al.* previously showed that a mixture of soluble egg antigens of *S. mansoni* (*Sm*SEA) could not protect mice from acute dextran sulfate sodium (DSS) colitis, whereas *S. mansoni* infection did and both treatments provoked mucosal Th2 responses.[14] Here we tested the therapeutic potential of a repeated treatment with *Sm*SEA in a chronic colitis model in mice. We focused on the anti-inflammatory properties of *Sm*SEA and investigated the immunological T cell mechanisms underlying the antigen-induced effects.

MATERIALS AND METHODS

Mice

C.B.-17 SCID and BALB/c mice were obtained from Charles River (L'Arbresle Cedex, France) and maintained in individually ventilated cages. All mice were female and 8 to 9 weeks of age at the initiation of the experiments.

Chronic adoptive T cell transfer colitis model

Colitis was induced in immunocompromised SCID mice by the adoptive transfer of $CD4^+CD25^-CD62L^+$ T cells as described previously.[15] Briefly, $CD4^+CD25^-CD62L^+$ T cells were isolated from the spleens of BALB/c donor mice using a magnetic $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To induce colitis, 1 x 10^6 $CD4^+CD25^-CD62L^+$ T cells in 100 µl phosphate-buffered saline (PBS) were intraperitoneally (i.p.) transferred into SCID mice. Two to three weeks after this adoptive transfer, SCID mice started developing colitis. Control mice were injected i.p. with 100 µl PBS and did not develop colitis.

Preparation of *S. mansoni* soluble egg antigens

S. mansoni soluble egg antigens (*Sm*SEA) were prepared as described previously with slight modifications.[16] Briefly, eggs were harvested from the livers of *S. mansoni*-infected mice. Livers were homogenized using an Ultra-Turrax, and eggs were separated from liver tissue by passing the liver homogenate through sieves. The eggs were collected in PBS on ice and washed 10 times by centrifugation at 1200 rpm, 2 min, 4°C with PBS. Collected samples were controlled by microscopy to ensure that they contained eggs only.

*Sm*SEA were prepared by homogenizing the eggs in a small volume of PBS using a glass homogenizer. The soluble fraction was collected after centrifugation and stored at -80°C. All steps were performed under sterile conditions in a workbench with sterile, LPS-free materials and solutions. Protein concentration was determined by MicroBCA-Kit (Thermo Scientific, Waltham, MA, USA), and endotoxin content by LAL-Haemochrom-Assay (Cape Cod Inc., East Falmouth, MA, USA).

Experimental design

In a first study protocol, SCID mice were treated once weekly (on Mondays) with vehicle (PBS) or *Sm*SEA in a dose of 20 µg/week (i.p.) during 5 weeks and starting two weeks after the adoptive transfer with $CD4^+CD25^-CD62L^+$ T cells or PBS (Fig. 1A). In a second study protocol, SCID mice were treated twice a

week (on Mondays and Thursdays) with vehicle (PBS) or *Sm*SEA in a dose of 20 µg/injection (i.p.) during 3 weeks and starting two weeks after the adoptive transfer with $CD4^+CD25^-CD62L^+$ T cells or PBS (Fig. 1B). The following groups were included in the studies: control mice treated with PBS (CONTROL), control mice treated with *Sm*SEA (CONTROL+*Sm*SEA), colitis mice treated with PBS (COLITIS) and colitis mice treated with *Sm*SEA (COLITIS+*Sm*SEA).

During the treatment periods, mice were monitored longitudinally via body weight measurements, a clinical disease score and endoscopic examinations of the colon.[15] At the end of the studies (week 6 for study 1 and week 4 for study 2), mice were sacrificed for the post-mortem macroscopic examination of the colon. To examine the underlying immunological mechanisms of *Sm*SEA, flow cytometric T cell characterization was performed on the lamina propria mononuclear cells (LPMC) isolated from the colon and cytokine concentrations in colon supernatants were measured via enzyme-linked immunosorbent assay (ELISA) or cytometric bead array (CBA) (Fig. 1).

Clinical examination

At different timepoints during the treatment periods (each time on Wednesdays at weeks 2, 3, 4, 5 and 6 for study 1 and at weeks 2, 3 and 4 each time for study 2), SCID mice were weighed and a clinical disease score was given to individual mice based on the following parameters: body weight, pilo-erection, stool consistency and physical activity.[15] Each parameter was graded from 0 to 2 according to the severity of disease. The cumulative score ranged from 0 (no clinical signs of illness) to 8 (severe clinical signs of illness).

Endoscopic examination of the colon

For the continuous monitoring of colitis (each time on Wednesdays at weeks 2, 4 and 6 for study 1 and at weeks 2 and 4 for study 2), colonoscopy was performed as described previously using a flexible Olympus URF type P5 ureteroscope with outer diameter of 3.0 mm and a 1.8 mm working channel (Olympus Europa GmbH, Hamburg, Germany).[15] In brief, mice were anesthetized with a mixture of ketamine (60 mg/kg, Ketalar; Pfizer, Puurs, Belgium) and xylazine (6.67 mg/kg, Rompun; Bayer, Leverkusen, Germany) (i.p.) and placed in a supine position. The anal sphincter and endoscope were lubricated with gel (RMS-Endoscopy, St. Martens-Lennik, Belgium) to facilitate the insertion of the endoscope. The endoscope was carefully introduced through the anus into the sedated mouse and under video guidance further inserted into the colon as far as possible. During the withdrawal of the endoscope, an endoscopic scoring was performed. The colonoscopic grading scale was based on the following parameters: colonic translucency,

the presence of fibrin attached to the bowel wall, the morphology of the vascular pattern, and the presence of loose stools (scored each between 0 and 3). The cumulative score ranged from 0 (no signs of inflammation) to 12 (signs of severe inflammation).

Macroscopic examination of the colon

At the end of the experiments (week 6 for study 1 and week 4 for study 2), mice were sacrificed by exsanguination under anesthesia and their colons were removed to score the colonic mucosal damage macroscopically as described previously.[15] The macroscopic inflammation score included the following 4 parameters: ulcerations, hyperemia, bowel wall thickening and mucosal edema. Each parameter was given a score from 0 (normal) to 3 (severe), leading to a cumulative score ranging from a minimum of 0 (no signs of inflammation) to a maximum of 12 (signs of severe inflammation).

Isolation of LPMC

Colonic LPMC were isolated as described previously.[17, 18] In order to obtain a sufficient amount of LPMC for the flow cytometric analysis, colons had to be pooled from two mice. Colon tissue was opened longitudinally, cut into 5 mm pieces and incubated in 30 ml calcium- and magnesium- free Hank's balanced salt solution (HBSS) containing 0.5 mM EDTA and 2 mM DL-dithiothreitol for 20 min at 37°C. Incubations were repeated after thorough washing. Tissue was then incubated for 20 min at 37°C in 20 ml lymphocyte growth medium (RPMI 1640 medium containing 10% fetal calf serum (FCS), 25 mM HEPES buffer, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 1 mM sodium pyruvate, 1% MEM non-essential amino acids 100x, 2% MEM amino acids 50x, 100 U/ml penicillin and 100 µg/ml streptomycin) containing 2 mg/ml collagenase (all from Life Technologies Europe (Gent, Belgium) or Sigma-Aldrich (St. Louis, MO, USA)). At the end of the incubation period the tissue was subjected to further mechanical disruption using a 1 ml syringe. To remove debris, the cell suspension was washed through prewetted gauze, layered in a funnel with lymphocyte growth medium. Then the LPMC were washed once and sieved through a prewetted 4 cm nylon wool column. After washing, cells (up to 2 x 10^7) were layered onto a 30:70% gradient Percoll column. Cells were spun at 3500 rpm for 20 min. The LPMC collected from the 30:70 interface were washed and maintained on ice until used.

Flow cytometric T cell characterization of LPMC

Three different flow cytometric stains were performed on LPMC: a general stain, a stain for regulatory T cells and a stain for intracellular cytokines.

General stain: LPMC were stained with anti-CD4 PerCP-Cy5.5 (clone: RM4-5; BD Biosciences, Erembodegem, Belgium) and anti-CD3 FITC (clone: 17A2; BD Biosciences).

Regulatory T cell stain: LPMC were stained with anti-CD4 PerCP-Cy5.5 (clone: RM4-5; BD Biosciences) and anti-CD25 FITC (clone: 7D4; BD Biosciences). Then cells were fixed and permeabilized with the Foxp3 Stainig Buffer Set® (eBioscience, San Diego, CA, USA) and stained with anti-Foxp3 AF645 (clone: MF23; BD Biosciences).

Intracellular cytokine stain: prior to staining, LPMC were cultured for 4 h at 37°C and 5% CO_2 in lymphocyte growth medium containing phorbol myristate acetate (1 µg/ml), ionomycin (1 µg/ml) and brefeldin A (10 µg/ml) (all from Sigma-Aldrich, St. Louis, MO, USA). After 4 h of incubation, the cells were stained with anti-CD4 PerCP-Cy5.5 (clone: RM4-5; BD Biosciences). Then LPMC were fixed with BD Cytofix® buffer (BD Biosciences) and permeabilized with the BD Perm/Wash® buffer (BD Biosciences). Next LPMC were stained with anti-IL-17A AF488 (clone: TC11-18H10; BD Biosciences), anti-IL-4 PE (clone: 11B11; BD Biosciences) and anti-IL-10 APC (clone: JES5-16E3; BD Biosciences).

LPMC were examined on a BD Accuri C6 (BD Biosciences, Erembodegem, Belgium) and analyzed using FCS Express 4 software (De Novo Software, Los Angeles, CA, USA). The different cell populations were gated using fluorescence minus one (FMO) controls. The gating strategy is shown in Fig. 2.

Colon cultures

A full thickness tissue sample was harvested from the colon in a standardized way and incubated for 24 h at 37°C and 5% CO_2 in complete RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Life Technologies Europe, Gent, Belgium). Colon supernatants were collected after 24 h and stored at -80°C for ELISA or CBA analyses.

ELISA

The concentration of IL-17A, IL-4 and IL-10 in colon supernatants were assayed by four-member solid phase sandwich ELISA according to the manufacturer's instructions (Life Technologies Europe, Gent, Belgium).

CBA

The levels of IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A and IL-10 were measured in the colon supernatants samples using the BD® CBA Mouse Th1/Th2/Th17 Cytokine Kit according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium).

Presentation of results and statistical analysis

Data are presented as mean ± SEM, for "n" representing the number of mice. The generalized estimating equation model was used to analyze the evolution of body weight, the clinical disease score and the colonoscopic score with time, followed by a least significant difference (LSD) post hoc analysis when appropriate. Two-way ANOVA followed by one-way ANOVA with LSD post hoc analysis was used when appropriate to compare the results of the macroscopic score, flow cytometry, ELISA and CBA between groups. An unpaired Student's *t* test was performed to compare the flow cytometric data between the COLITIS and the COLITIS+*Sm*SEA groups. *P* values of ≤ 0.05 were considered as significant. Data were analyzed using SPSS 18.0 software and GraphPad Prism 5.00.

ETHICAL CONSIDERATIONS

All animal experiments were performed in accordance with the guidelines of the Committee for Medical Ethics and the use of Experimental Animals at the University of Antwerp that approved the study protocol (file number 2010-28).

RESULTS

Effect of *Sm*SEA on the inflammatory parameters during experimental colitis (study 1)

CONTROL mice treated with PBS or *Sm*SEA gained weight during the treatment period, whereas the body weight of COLITIS mice treated with PBS significantly decreased between week 2 and week 5 (Fig. 3A). *Sm*SEA treatment stabilized the colitis-induced loss of body weight of COLITIS+*Sm*SEA mice from week 4 onward (Fig. 3A).

The clinical disease score of COLITIS mice significantly increased between week 2 and week 4 and remained stable from week 4 onward (Fig. 3B). In COLITIS+*Sm*SEA mice, the administration of the egg antigens resulted in a significant lower clinical disease score from week 3 onward compared with the clinical disease score of COLITIS mice (Fig. 3B). However, this protective effect of *Sm*SEA diminished with time as only a tendency ($P = 0.06$) remained in COLITIS+*Sm*SEA mice at the end of the study (week 6) (Fig. 3B). CONTROL mice and CONTROL+*Sm*SEA mice showed no clinical signs of illness (Fig. 3B).

The endoscopic examination of the colon revealed no colonoscopic signs of inflammation in CONTROL and CONTROL+*Sm*SEA mice (Fig. 3C). On the contrary, the signs of mucosal inflammation in COLITIS mice gradually increased over time as evidenced by the significant increase of the colonoscopic score (Fig. 3C). The colonoscopic score of COLITIS+*Sm*SEA mice was significantly lower at week 2 (this is 2 days after the first *Sm*SEA injection) and remained significantly lower from week 2 onward compared with the colonoscopic score of COLITIS mice (Fig. 3C). Colonoscopy confirmed that the beneficial effect of *Sm*SEA treatment decreased with time (Fig. 3C).

At the end of this first study (week 6), mice were sacrificed for the post-mortem macroscopic examination of the colon. CONTROL and CONTROL+*Sm*SEA mice showed no macroscopic sign of inflammation (Fig. 3D). The mucosal damage scored during the macroscopic examination was not statistically different between COLITIS and COLITIS+*Sm*SEA mice (Fig. 3D).

Effect of *Sm*SEA on the immunological response in the colon during experimental colitis (study 1)

To examine the underlying immunological mechanisms of *Sm*SEA treatment in this study, flow cytometric T cell characterization was performed on the LPMC and colonic cytokine concentrations were measured via ELISA on the colon supernatants.

In order to obtain a sufficient amount of LPMC for flow cytometry, colons had to be pooled from two mice. Flow cytometric T cell characterization of the LPMC showed an equal upregulation of $CD4^+$ cells between COLITIS and COLITIS+*Sm*SEA mice (Fig. 4A). As expected no $CD4^+$ cells were upregulated in CONTROL and CONTROL+*Sm*SEA mice as SCID mice are immunodeficient (Fig. 4A). For that reason, further T cell characterization was only performed for COLITIS and COLITIS+*Sm*SEA mice. Within the population of $CD4^+$ LPMC, almost all cells expressed CD3 both in the COLITIS and COLITIS+*Sm*SEA mice. This indicates that the gated $CD4^+$ LPMC are actually $CD4^+CD3^+$ effector T cells (Fig. 4B). Within this population of effector T cells, the administration of *Sm*SEA did not affect the amount of cells expressing CD25 and Foxp3 (probably Treg cells) and it did not significantly affect the amount of cells producing IL-17A (Th17 cells), IL-4 (Th2 cells) and IL-10 (Treg, Th2 and/or Th9 cells), as comparable percentages of these T cell subsets were found in COLITIS and COLITIS+*Sm*SEA mice (Fig. 4C-F).

These flow cytometric results were confirmed by ELISA of the colon supernatants (table 1): comparable concentrations of IL-17A and IL-10 were found in COLITIS and COLITIS+*Sm*SEA mice. In the colon supernatants of CONTROL and CONTROL+*Sm*SEA mice none of these cytokines could be detected. IL-4 could hardly be detected in any sample.

Effect of *Sm*SEA on the inflammatory parameters during experimental colitis (study 2)

CONTROL mice that were treated twice a week with PBS or *Sm*SEA gained weight during the treatment period, whereas the body weight of COLITIS mice treated with PBS significantly decreased between week 2 and week 4 (Fig. 5A). The body weight of COLITIS+*Sm*SEA mice also decreased during the treatment period. However, *Sm*SEA treatment appeared to stabilize the colitis-induced loss of body weight between week 3 and week 4 in the COLITIS+*Sm*SEA mice (Fig. 5A).

The clinical disease score of COLITIS mice significantly increased with time (Fig. 5B). Administration of *Sm*SEA however stabilized the colitis-induced clinical signs of illness in COLITIS+*Sm*SEA mice. The clinical disease score of these mice was significantly lower compared with the clinical disease score of COLITIS mice from week 3 onward (Fig. 5B). CONTROL and CONTROL+*Sm*SEA mice showed no clinical signs of illness (Fig. 5B).

The colonoscopic score of both the COLITIS and COLITIS+*Sm*SEA mice significantly increased with time (Fig. 5C). A slightly lower colonoscopic score was found at week 4 in the COLITIS+*Sm*SEA mice compared

with COLITIS mice (5.6 ± 0.7 and 7.1 ± 0.7 respectively), but statistical significance was not reached (Fig. 5C). CONTROL and CONTROL+*Sm*SEA mice did not show any colonoscopic signs of inflammation (Fig. 5C).

The administration of *Sm*SEA tended to improve the mucosal damage in the colons of the COLITIS+*Sm*SEA mice at the end of this second study (week 4) ($P = 0.08$), as evidenced by a slightly lower macroscopic inflammation score compared with COLITIS mice (6.9 ± 0.7 and 5.3 ± 0.8 respectively) (Fig. 5D). CONTROL and CONTROL+*Sm*SEA mice did not shown any macroscopic signs of inflammation (Fig. 5D).

Effect of *Sm*SEA on the immunological response in the colon during experimental colitis (study 2)

The immunological mechanisms underlying the protective effect of the *Sm*SEA therapy in this second study was examined via flow cytometric T cell characterization of the LPMC and via CBA of the colon supernatants to determine colonic cytokine concentrations. In this study, ELISA analyses were replaced by CBA as CBA could quantify multiple cytokines simultaneously in one analysis.

Flow cytometric T cell characterization of the LPMC showed equal amounts of $CD4^+$ cells in the COLITIS and COLITIS+*Sm*SEA mice (Fig. 6A). No $CD4^+$ LPMC were found in CONTROL and CONTROL+*Sm*SEA mice as expected (Fig. 6A). Within the population of $CD4^+$ LPMC of both the COLITIS and COLITIS+*Sm*SEA mice, almost all cells expressed CD3, indicating that the gated $CD4^+$ LPMC are $CD4^+CD3^+$ effector T cells (Fig. 6B). Further analyses of these effector T cells revealed comparable percentages of cells expressing CD25 and Foxp3 (probably Treg cells) between COLITIS and COLITIS+*Sm*SEA mice (Fig. 6C). Interestingly the administration of *Sm*SEA significantly downregulated the amount of IL-17A-producing effector T cells (Th17 cells) and significantly upregulated the amount of IL-4-producing effector T cells (Th2 cells) in COLITIS+*Sm*SEA mice compared with COLITIS mice (Fig. 6D,E). The amount of IL-10-producing effector T cells (Treg, Th2 and/or Th9 cells) was comparable between COLITIS+*Sm*SEA and COLITIS mice ($P = 0.125$) (Fig. 6F).

CBA analysis of colon supernatants showed higher levels of the proinflammatory cytokines IL-2, TNF, IFN-γ, IL-6 and IL-17A and higher levels of the anti-inflammatory cytokine IL-10 in COLITIS and COLITIS+*Sm*SEA mice compared with CONTROL and CONTROL+*Sm*SEA mice (table 1). The increase of IL-2, TNF, IL-6 and IL-10 was less pronounced in COLITIS+*Sm*SEA mice compared with COLITIS mice, resulting

in a loss of significance between COLITIS+*Sm*SEA mice and CONTROL/CONTROL+*Sm*SEA mice (table 1). No significant difference was seen in the concentration of IL-4 between all animal groups (table 1).

DISCUSSION

We investigated the therapeutic anti-inflammatory potential of *S. mansoni* soluble egg antigens (*Sm*SEA) on experimental colitis. Here we provide evidence that *Sm*SEA reduce the severity of experimental colitis in the adoptive T cell transfer mouse model. Our results point towards a role of Th2 cells in the underlying immunological mechanism involved in the protective effect of *Sm*SEA.

To evaluate the effect of *Sm*SEA on the clinical signs of illness and on the colonic inflammation over time, body weight changes were recorded, a clinical disease score was given and endoscopic examinations were performed on the colon. These three assessment techniques allowed us to monitor mice during the entire experiment without the need to kill the mice.[15, 19]

The colitis in the adoptive $CD4^+CD25^-CD62L^+$ T cell transfer model caused a significant increase in all the inflammatory parameters studied. In the first study, a weekly administration of *Sm*SEA during a period of 5 weeks ameliorated the clinical signs of illness and the intestinal inflammation of mice with colitis as evidenced by a significant reduction of the clinical disease score and the colonoscopic score and by a stabilization of the body weight loss. In this study however, the protective effect of *Sm*SEA diminished with time as the significant difference between *Sm*SEA-treated and PBS-treated colitis mice disappeared (for the clinical disease score), became less pronounced (for the colonoscopic score) or was not reached (for the macroscopic score) by the end of the study (week 6).

We therefore designed a second study, in which mice were treated twice a week. Based on the beneficial effects of *Sm*SEA which were apparent in the earlier timepoints in study 1, the decision was made to sacrifice the mice in this second study at week 4 to examine the colonic inflammation and to investigate the immunological response. At week 4 of this study, the administration of *Sm*SEA to mice with colitis resulted in a significant decrease in the clinical disease score and a less severe loss in body weight. However, at this timepoint the colonoscopic score was only minimally affected by *Sm*SEA treatment. In this second study, the administration of *Sm*SEA to colitis mice caused a tendency towards a lower macroscopic inflammation score. Based on these results, we can say that treating the animals twice a week with *Sm*SEA does not necessarily result in a better outcome compared to treating once weekly.

The administration of *Sm*SEA to control mice did not affect the inflammatory parameters studied in these mice and did not cause an altered immune response as confirmed in both studies.

To unravel the immunological mechanisms underlying the anti-inflammatory effect of *Sm*SEA, and in particular the balance between Th1, Th17, Th2 and Treg cells, flow cytometric T cell characterization was performed on the LPMC and colonic cytokine concentrations were measured via ELISA and CBA in colon supernatants.

At the end of the first study (week 6), *Sm*SEA treatment did not affect the amount of $CD4^+$ effector T cells expressing CD25 and Foxp3 (probably Treg cells), producing IL-17A (Th17 cells), producing IL-4 (Th2 cells) or producing IL-10 (Treg, Th2 and/or Th9 cells), and this was also confirmed by ELISA analyses on colon supernatants. This lack of long-lasting immune responses could be a logical consequence of the gradually declining protective effect of *Sm*SEA seen by the end of the study. The lack of B cells and consequently the lack of IgE and regulatory B (Breg) cells in the immunocompromised SCID mice might also explain this declining protective effect of *Sm*SEA (personal communication of dr. Gabriele Schramm).[20, 21] The Th2 response in the host induced by molecules present in SmSEA such as omega-1 and IPSE/alpha-1, consists of a biphasic Th2 response with an initial IgE-independent mechanism followed by an IgE-dependent activation of the Th2 response.[6, 12, 22] We therefore hypothesize that since SCID mice do not develop these secondary antibody responses, due to the lack of B cells, the protective effect of *Sm*SEA will not be sustained and thus no long-lasting anti-inflammatory response can be present in these SCID mice. Another possible explanation could relate to an insufficient dosage regimen. For example, we previously could show a small therapeutic window for other *S. mansoni*-derived molecules, namely *S. mansoni* soluble worm proteins (*Sm*SWP), indicating the importance of finding the right dosage when testing the therapeutic potential of helminth-derived molecules.[18]

The flow cytometric T cell characterization of the LPMC in the second study indeed showed that treatment of colitis mice with *Sm*SEA downregulated the colonic proinflammatory Th17 response while causing an upregulation of a Th2 response at week 4. It also showed that the Treg responses were not affected after the *Sm*SEA treatment at week 4. Our flow cytometric results thus suggest a downregulation of the proinflammatory immune response but CBA analysis on colon supernatants only partially confirmed this observation. The concentrations of the proinflammatory cytokines IL-2, TNF and IL-6, but not IFN-γ and IL-17A, were downregulated in the colon supernatants of *Sm*SEA-treated colitis mice compared with PBS-treated colitis mice, resulting in a loss of significance between *Sm*SEA-treated colitis mice and controls. The Th2 response (upregulated IL-4) detected by flow cytometry in colonic LPMC was not confirmed with the CBA analysis on colon supernatants. This might be related to the fact that it was proven in the past that IL-4 is a difficult cytokine to measure in culture supernatant, as it can

be rapidly consumed in the supernatants.[23, 24] Besides, previously it was reported in literature that cytokine assays such as ELISA or CBA not necessarily parallel the intracellular data of flow cytometry.[24] The latter was confirmed in this second study, as IL-4-producing effector T cells were found in higher numbers in the LPMC of *Sm*SEA-treated colitis mice despite the detection of low levels of IL-4 in the colon supernatants. In contrast, IL-17A-producing effector T cells were found at low levels in the LPMC of *Sm*SEA-treated colitis mice despite the high levels of IL-17A in culture supernatants. So in this second study, the cytokine phenotype of particular cell types detected via flow cytometry on LPMC was not incontrovertibly reflected by the concentration of the respective cytokines in the colon supernatants measured via CBA. This is well in line with the suggestion that, in order to obtain the most accurate immunological picture, different methods of cytokine detection need to be used.[24] Moreover, the use of colonic supernatants instead of colonic LPMC might play a role in the differences between our CBA data and flow cytometric data, as cytokines first need to be secreted in the colonic supernatants before they can be detected via CBA. The method for the intracellular stain for flow cytometry typically includes a step in which LPMC are stimulated to produce cytokines, whereas the method to obtain supernatants from colonic tissues, used for the CBA cytokine analysis, does not include such an additional stimulation step representing another important difference in the methodology.

Our flow cytometric data thus confirm the hypothesis that *Sm*SEA treatment can restore the disturbed intestinal immune balance through the induction of Th2 responses that drive the downregulation of the proinflammatory Th17 responses. So our data demonstrate a substantial role of Th2 cells in the underlying immunological mechanism involved in the protective effect of *Sm*SEA. Other research groups defined possible cellular components of the innate immune system that might be involved in this immunological mechanism of Th2 induction by *Sm*SEA. For example, Schramm *et al.* reported that *Sm*SEA trigger the release of the Th2 cytokines IL-4 and IL-13 from basophils in an IgE-dependent way.[6, 12, 22] This effect was mainly mediated by the glycoprotein IPSE/alpha-1, the most abundant molecule present in *Sm*SEA.[25, 26] However, since the immunocompromised SCID mice used in our study lack B cells, we can assume that the mechanism underlying the anti-inflammatory effect of *Sm*SEA seen in this study is not IgE-dependent. These results indicate that *Sm*SEA exert their immunomodulatory effects through different mechanisms which may also involve dendritic cells (DCs) and/or macrophages. Everts *et al.* reported, that the glycoprotein omega-1, a molecule present in *Sm*SEA, drives Th2 responses *in vitro* via the functional modulation of DCs.[5, 10] The mechanisms involved in the functional modulation of DCs are a decreased expression of the co-stimulatory molecules CD80 and CD86, a reduced production of the

proinflammatory cytokine IL-12 and an impaired DC-T cell interaction. Omega-1 alone displayed the same Th2-inducing potency as *Sm*SEA and it was shown *in vivo* to drive Th2 responses with similar characteristics as *Sm*SEA.[5, 6, 10, 25] Furthermore, it was also shown that lacto-N-fucopentaose III, another molecule present in *Sm*SEA, drives Th2 responses through the alternative activation of macrophages.[8, 9, 11]

In conclusion, we could demonstrate that the administration of *Sm*SEA reduced the severity of colitis in the adoptive T cell transfer mouse model, as shown by the improvement of the inflammatory parameters studied. It was proven that the anti-inflammatory effect of *Sm*SEA was initiated by the induction of a Th2 response which suppressed the proinflammatory Th17 response in the colon. However, the maximal immunological response seems to precede the maximal anti-inflammatory response. While focusing on the T cell responses of the adaptive immune system during *Sm*SEA therapy, other research groups identified other cells of the adaptive and innate immune system to be important in the *Sm*SEA-induced polarization of Th2 cells such as B cells, basophils, macrophages and dendritic cells. This indicates that *Sm*SEA activate several distinct regulatory pathways involving cellular components of both the innate and adaptive immune system to control gut inflammation.

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TABLE LEGEND

TABLE 1. Cytokine profiles in colon supernatants measured by ELISA (study 1) and CBA (study 2).

FIGURE LEGENDS

FIGURE 1. Schematic presentation of the experimental design. At week 0, colitis was induced in immunocompromised SCID mice by the i.p. injection of $CD4^+CD25^-CD62L^+$ T cells, controls were injected with PBS. In study 1 **(A)**, mice were treated once weekly (on Mondays) with 20 µg/week *Sm*SEA or vehicle (PBS) starting two weeks after T cell or PBS injections. In study 2 **(B)**, mice were treated twice a week (on Mondays and Thursdays) with 20 µg/injection *Sm*SEA or vehicle (PBS) starting two weeks after T cell or PBS injections. During the treatment periods, body weight changes were recorded, a clinical disease score was given and colonoscopy was performed at different timepoints, but each time on Wednesdays. At the end of the studies (week 6 for study 1 and week 4 for study 2), mice were sacrificed for the post-mortem examination of the colon and for the examination of the underlying immunological mechanisms of *Sm*SEA.

CD, cluster of differentiation; i.p., intraperitoneally; PBS, phosphate-buffered saline, *Sm*SEA: *Schistosoma mansoni* soluble egg antigens.

FIGURE 2. Multi-color flow cytometric gating strategies for the T cell characterization of LPMC. Gating strategy for the general stain **(A)**: $CD4^+$ cells were initially gated upon the expression of CD4 and SSC properties, and subsequently gated and assessed upon the expression of CD3. Gating strategy for the regulatory T cell stain **(B)**: $CD4^+$ cells were initially gated upon the expression of CD4 and SSC properties, and subsequently gated and assessed upon the expression of CD25 and Foxp3. The gating strategy for the intracellular cytokine stain **(C)**: $CD4^+$ cells were initially gated upon the expression of CD4 and side SSC properties, and subsequently gated and assessed upon the expression of IL-17A, IL-4 or IL-10.

CD, cluster of differentiation; Foxp3, forkhead box p3; IL, interleukin; LPMC, lamina propria mononuclear cells; SSC, side scatter.

FIGURE 3. Study 1: Effect of *Sm*SEA on the inflammatory parameters. Effect on body weight **(A)**, clinical disease score **(B)**, colonoscopic score **(C)** and the macroscopic inflammation score at week 6 **(D)**. Data are presented as mean ± SEM. Generalized Estimations Equations were used to analyze the evolution of body weight, clinical disease score and colonoscopic score over time and a LSD post hoc analysis was applied. Two-way ANOVA was used to compare the results of macroscopy between groups. $^{\#}$: $P \leq 0.05$, significant difference between CONTROL/CONTROL+*Sm*SEA and COLITIS/COLITIS+*Sm*SEA groups; *: $P \leq 0.05$, a significant difference between the COLITIS and COLITIS+*Sm*SEA groups; §: $P \leq 0.05$, significant increase of score with time within groups; "n" representing the number of mice.

LSD, least significant difference; SEM, standard error of the mean*; Sm*SEA, *Schistosoma mansoni* soluble egg antigens.

FIGURE 4. Study 1: Flow cytometric T cell characterization of the LPMC after *Sm*SEA treatment. % of $CD4^+$ cells **(A)**, % of $CD4^+$ cells expressing CD3 **(B)**, % of $CD4^+$ T cells expressing CD25 and Foxp3 (probably Treg cells) **(C)**, % of $CD4^+$ T cells producing IL-17A **(D)**, % of $CD4^+$ T cells producing IL-4 **(E)** and % of $CD4^+$ T cells producing IL-10 **(F)**. Data are presented as mean ± SEM. Two-way ANOVA or an unpaired Student's *t* test was used as appropriate to compare the flow cytometric results between groups. $^{\#}$: $P \leq 0.05$, significant difference between CONTROL/CONTROL+*Sm*SEA and COLITIS/COLITIS+*Sm*SEA group; "n" representing the number of mice.

CD, cluster of differentiation; Foxp3, forkhead box p3; IL, interleukin; LPMC, lamina propria mononuclear cells; %, percentage; SEM, standard error of the mean; *Sm*SEA, *Schistosoma mansoni* soluble egg antigens.

FIGURE 5. Study 2: Effect of *Sm*SEA on the inflammatory parameters. Effect on body weight **(A)**, clinical disease score **(B)**, colonoscopic score **(C)** and macroscopic inflammation score at week 4 **(D)**. Data are presented as mean ± SEM. Generalized Estimations Equations were used to analyze the evolution of body weight, clinical disease score and colonoscopic score over time and a LSD post hoc analysis was applied. Two-way ANOVA was used to compare the results of macroscopy between groups. $^{\#}$: $P \leq 0.05$, significant difference between CONTROL/CONTROL+*Sm*SEA and COLITIS/COLITIS+*Sm*SEA groups; *: $P \leq 0.05$, a significant difference between the COLITIS and COLITIS+*Sm*SEA groups; §: $P \leq 0.05$, significant increase of score with time within groups; "n" representing the number of mice.
LSD, least significant difference; SEM, standard error of the mean; *Sm*SEA, *Schistosoma mansoni* soluble egg antigens.

FIGURE 6. Study 2: Flow cytometric T cell characterization of the LPMC after *Sm*SEA treatment. % of $CD4^+$ cells **(A)**, % of $CD4^+$ cells expressing CD3 **(B)**, % of $CD4^+$ T cells expressing CD25 and Foxp3 (probably Treg cells) **(C)**, % of $CD4^+$ T cells producing IL-17A **(D)**, % of $CD4^+$ T cells producing IL-4 **(E)** and % of $CD4^+$ T cells producing IL-10 **(F)**. Data are presented as mean ± SEM. Two-way ANOVA or an unpaired Student's *t* test was used as appropriate to compare the flow cytometric results between groups. $^{\#}$: $P \leq 0.05$, significant difference between CONTROL/CONTROL+*Sm*SEA and COLITIS/COLITIS+*Sm*SEA group; *: $P \leq 0.05$, a significant difference between COLITIS and COLITIS+*Sm*SEA groups; "n" representing the number of mice.

CD, cluster of differentiation; Foxp3, forkhead box p3; IL, interleukin; LPMC, lamina propria mononuclear cells; %, percentage; SEM, standard error of the mean; *Sm*SEA, *Schistosoma mansoni* soluble egg antigens.

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