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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Effect of regulatory T cells on the efficacy of the fatty acid-binding protein vaccine against *Schistosoma japonicum*

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Abstract

Schistosomiasis is one of the most devastating parasitic diseases, making it imperative to develop efficient vaccines to control the causative flatworms called schistosomes. Regulatory T cells (Tregs) and the Th1 immune response have been implicated in the effectiveness of vaccines to control schistosomiasis, but the mechanisms underlying their effects are unclear. In this study, we evaluated the role of Tregs on the efficacy of the 14 kDa FABP (fatty acid-binding protein) vaccine against *Schistosoma japonicum*. BALB/c female mice were randomly divided into five groups: an uninfected group, infected control group, anti-CD25 monoclonal antibody (anti-CD25 mAb) group, FABP group, and combined anti-CD25 mAb and FABP group. Compared with FABP alone, a combined treatment with FABP and anti-CD25 mAb increased the rate of *S. japonicum* inhibition in mice from 30.3 to 56.08% and decreased the number of eggs per gram of liver. Compared with that of the infected control group, the percentage of Tregs in the spleen decreased significantly after single or combined treatment with FABP and anti-CD25 mAb, while it increased gradually in the anti-CD25 mAb group. Further, the secretion of Th1 cytokines, IFN-γ, and IL-2 increased in splenocytes in the anti-CD25 mAb group. Our results indicate that anti-CD25 mAb partially blocks Tregs and concomitantly enhances the Th1 type immune response, thereby enhancing the protective effect of the FABP vaccine.

Keywords *Schistosoma japonicum* · Fatty acid-binding protein · Regulatory T cells · Anti-CD25 mAb · Cytokines

Introduction

The World Health Organization recommends six kinds of vaccines against *Schistosoma japonicum*, including fatty acid-binding proteins (FABPs), which display various degrees of therapeutic effects. *Schistosoma* glyceraldehyde-3-p-dehydrogenase, a surface antigen, is a candidate subunit vaccine for inducing protective immunity against schistosomiasis (Perez-Casal and Potter 2016). In the case of *S. mansoni* infection, immunization with the 14-3-3 protein leads to protection ranging from 25 to 46%, as determined by a reduction in the adult worm burden (Schechtman et al. 2001). *Schistosoma* does not synthesize long-chain fatty acids and sterols, which must be extracted from the host, making *Sj*-FABP an attractive vaccine and/or drug target (Becker et al. 1994). In addition, owing to its structural similarity to a *Fasciola hepatica* antigen, *S. mansoni* Sm14 FABP has been tested as a potential vaccine against both schistosomiasis and fascioliasis (Almeida et al. 2003; Tendler et al. 1996). Likewise, the *F. hepatica* FABP protein has a high protective effect against *S. mansoni* infection (Vicente et al. 2016). Recently, the Sm14 successfully reached phase I clinical studies (Mossallam et al. 2015). Clinical trial data for the tolerance and specific immunoreaction of adult male volunteers with rSm14/GLA-SE demonstrate that FABP is a safe and strongly immunogenic vaccine against schistosomiasis, paving the way for future phase 2 trials (Santini-Oliveira et al. 2016). However, the mechanism

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of action of the vaccine is still unclear, and it is speculated that it may be associated with regulatory T cells (Tregs).

Several studies have highlighted the important role of Tregs in vaccine design and their protective effects. Peptide vaccine-induced antigen-specific Tregs attenuate antiviral immunity against influenza virus infection. Peptide vaccines with CpG-adjuvant may provide protection against influenza by inhibiting Treg development (Lin et al. 2018). It is necessary to shift the balance of T effector/T regulatory cells (Teff/Tregs) towards effectors to improve vaccine-specific immune responses for the design and development of HIV-1 therapeutic vaccines (Hubert and Seddiki 2018). Recently, we showed that *S. japonicum* GST vaccine had a poor protective effect, which could be enhanced by the addition of an anti-CD25 mAb that blocks Tregs (Tang et al. 2017). In the current study, to further examine the effect of Tregs on the efficacy of the *Sj*-FABP vaccine and the underlying mechanism, we blocked Tregs using an anti-CD25 mAb and observed the protective effect of FABP against *S. japonicum*.

Materials and methods

Animals and parasites

Fifty-four female BALB/c mice (6–8 weeks old) were purchased from the Hubei Province Center for Disease Control and Prevention, China, for experiments that were approved by the Animal Research Committee of Wuchang Hospital. The mice were randomly divided into five groups: an uninfected group, infected control group, anti-CD25 monoclonal antibody (anti-CD25 mAb) group, FABP group, and combined anti-CD25 mAb and FABP group. *Schistosoma japonicum*-infected snails were provided by the Jiangxi Province Parasitic Disease Control Institute. *S. japonicum* cercaria were shed from the snails.

Immunization and challenging experiments

The FABP used in this study is a recombinant protein prepared by our laboratory. Briefly, *Sj*-FABP full-length cDNA was amplified from the adult *S. japonicum* cDNA library using specific primers (forward: 5′-CCGGAATTCATGTC TTCTTTCTTGGGAA-3′; reverse: 5′-CCGC TCGAGACATTTGACCAGTCTATCA-3′. The PCR products of approximately 447 base pairs were ligated into the expression vector PET28α (+) (Novagen, Madison, WI, USA). The recombinant plasmid PET28α (+)-*Sj*-FABP was amplified in *Escherichia coli* DH5α cells and purified with the Axy PrepTM plasmid miniprep kit (Axygen, Hangzhou, China), followed by transformation into *E. coli* BL21 (DE3) cells. Positive colonies were screened by PCR and sequencing. After optimizing the culture conditions, 1 mM isopropyl-β-thiogalactopyranoside was added to induce *Sj*-FABP protein at 30 °C for 6 h, after which the OD 600 was approximately 0.5. The protein was purified using Ni-NTA Agarose (QIAGEN, Hilden, Germany) and quantified with a BCA protein Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol.

Mice in the FABP group and combination group were primed percutaneously with 50 μg of FABP against *S. japonicum*, which was emulsified with Freund's complete adjuvant in a 1:1 ratio. The same dosage of FABP emulsified 1:1 with incomplete adjuvant was boosted two times at 2-week intervals. The remaining three groups of mice received equal amounts of PBS. At 2 weeks after the last immunization, the remaining mice, except for normal control mice, were subcutaneously infected with 40 cercaria of *S. japonicum*. At 2 weeks post-infection, mice in the anti-CD25 mAb and co-treated groups received an intraperitoneal injection of 300 μg of anti-CD25 mAb (PC61; eBioscience, San Diego, CA, USA) or an equal volume of PBS as control (Tang et al. 2014). Mice in the anti-CD25 mAb group were sacrificed at 2 weeks, 2 weeks + 3 days, 3 weeks, and 4 weeks post-infection (i.e., 0 days, 3 days, and 1 and 2 weeks after anti-CD25 mAb administration) and was used for detection of Tregs by FACS. After 5 weeks of infection (3 weeks after anti-CD25 mAb administration), all mice were killed and examined.

Assessment of the numbers of worm and eggs

To evaluate the numbers of worms and eggs, mice in the four infected groups were killed and adult worms were obtained at 5 weeks post-infection, as described by Ruppel et al. (1990). Worms were counted under an anatomical microscope and the parasite reduction rate was calculated. The egg load in the liver was calculated according to Cheng et al. (2008) and mean values were obtained. Based on these, the number of eggs per gram of liver and reductions in egg burden were calculated.

Flow cytometry

According to protocol by Mo et al. (2008), a single-cell suspension of splenocytes at 5 weeks after infection was prepared to detect the percentage of Tregs. The Mouse Regulatory T Cell Staining Kit (eBioscience) was used and results were analyzed using the FACSCalibur (Becton Dickson, Franklin Lakes, NJ, USA) and Cell Quest software. The kit contained the following four antibodies: FITC-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD25, PE-conjugated anti-mouse Foxp3, and PE-conjugated rat IgG2α isotype control (eBioscience).

Splenocyte culture and analysis of mouse cytokines

At 5 weeks after infection, 6 mice in each group were killed to prepare spleen cell suspensions and 5×10^6 splenocytes/well were cultured in RPMI-1640 plus with 10% FCS and 1% penicillin and streptomycin (all from Sigma, St. Louis, MO, USA). *S. japonicum* soluble egg antigen (SEA) (5 μg/mL) was used to stimulate the growth and proliferation of spleen cells and the secretion of cytokines at 37 °C and 5% CO_2 for 72 h. ELISA was used to measure the levels of IFN-γ, IL-2, IL-4, and IL-5 in cell culture supernatants, according to the manufacturer's protocol (eBioscience). The MK3 microplate reader was used to detect OD values at 450 nm.

Statistical analysis

All data are expressed as means ± standard deviations and results were analyzed using SPSS 19.0. ANOVA was used to compare the differences among groups, and $P < 0.05$ was considered to be statistically significant.

Results

Protective effects of the anti-CD25 mAb on the FABP vaccine against *S. japonicum* in mice

To determine the protective effects of anti-CD25 mAb on the FABP vaccine against *S. japonicum* in mice, worms and eggs were counted in the four infected mice groups (6 mice per group) at 5 weeks after infection. The experiment was performed twice. Compared with those in the FABP alone or the anti-CD25 mAb alone groups, the number of adult worms in mice co-treated with FABP and anti-CD25 mAb was significantly lower ($P < 0.05$). The same trend was observed for the egg burden in the livers of co-treated mice (Table 1).

Effect of anti-CD25 mAb on the percentage of Tregs in the spleen

Because Foxp3 is a specific marker of Tregs (Ferreira et al. 2017), we used the percentages of $CD4^+CD25^+Foxp3^+$ T cells to evaluate Tregs in the spleens of all mice groups. To evaluate depletion by anti-CD25 mAb, infected mice were killed at five time points, namely, 0 day, 3 days, 1 week, 2 weeks, and 3 weeks post-anti-CD25 mAb administration. Representative FACS results for Tregs are shown in Fig. 1. At 3 days after the administration of the anti-CD25 mAb, the percentages of $CD4^+CD25^+Foxp3^+$ T cells in groups with anti-CD25 mAb decreased dramatically by more than 99%; thereafter, the percentages gradually rose.

Next, we determined whether Tregs are related to the protective efficacy of *Sj*-FABP vaccination. As shown in Fig. 2, the frequency of $CD4^+CD25^+Foxp3^+$ T cells increased after infection with *S. japonicum*. Compared with the infected control group, the frequencies of Tregs were significantly lower in anti-CD25 mAb group ($P = 0.012$), the FABP group ($P = 0.025$), and the co-treated group ($P = 0.000$). Compared with the co-treated control group, the frequencies of Tregs were significantly higher in anti-CD25 mAb ($P = 0.042$) and the FABP ($P = 0.031$) groups.

Cytokine production by splenocytes after FABP and anti-CD25 mAb treatment

As shown in Fig. 3, levels of IFN-γ, IL-2, IL-4, and IL-5 were compared among five groups. Based on ELISA, the levels of the Th1 cytokine IFN-γ were higher after the administration of anti-CD25 mAb alone ($P = 0.043$), FABP alone ($P = 0.038$), or combined treatment ($P = 0.000$), compared with the infected control group. Compared with the co-treated control group, the levels of IFN-γ were significantly lower in the anti-CD25 mAb group ($P = 0.028$) and the FABP group ($P = 0.030$). After the administration of anti-CD25 mAb ($P = 0.034$), FABP ($P = 0.029$), or combined treatment ($P = 0.000$), levels of the Th1 cytokine IL-2 were higher when compared with that in the infected control group. Compared with the co-treated control group, the levels of IL-2 were significantly lower in anti-CD25 mAb ($P = 0.037$) and FABP ($P = 0.026$) groups. However, there was no significant difference in the levels of the Th2 cytokines IL-4 and IL-5 among the groups. These results suggest that the anti-CD25 mAb improved the protective efficacy of the FABP vaccine against *S. japonicum* by enhancing the Th1 type immune response.

Table 1 Protective effects of the anti-CD25 mAb on the FABP vaccine against *S. japonicum* in mice

Group[n]	Worm burden	Worm reduction rate (%)	Eggs per gram of liver tissue (× 10^3)	Liver egg reduction rate (%)
Infected control	27.62 ± 2.80	/	21.86 ± 8.50	/
Anti-CD25 mAb	21.58 ± 2.63	21.87*	17.62 ± 6.80	19.39*
FABP	19.25 ± 2.53	30.30*	13.35 ± 4.50	38.90*
Co-treated	12.13 ± 1.46	56.08[#]	10.21 ± 3.30	53.29[#]

Note: *$P < 0.05$ compared with the infected control group. [#] $P < 0.05$ compared with the FABP group. Data were obtained from two independent experiments ($n = 6$ mice per group) and are presented as means ± SD

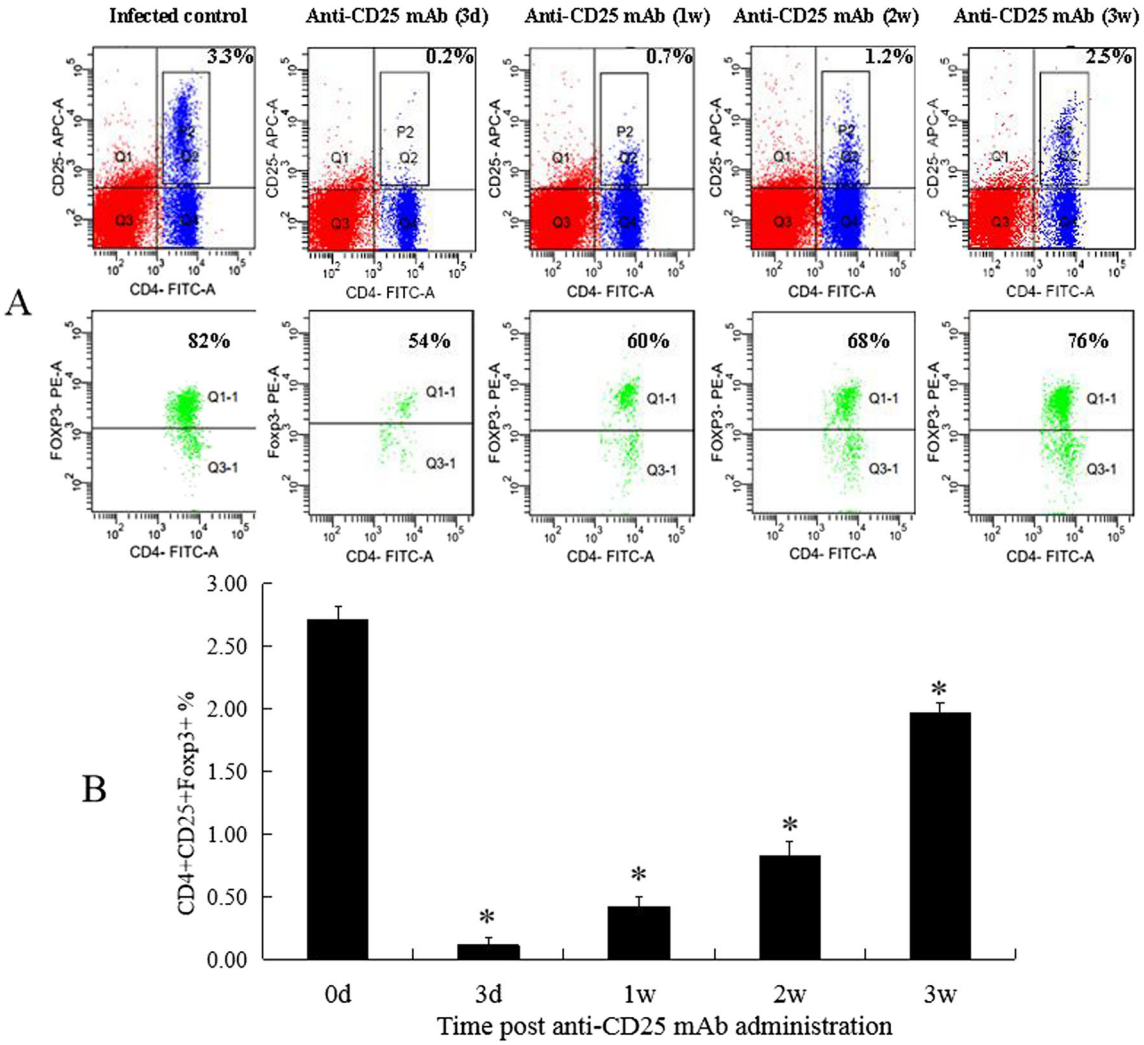

Fig. 1 **a** Representative FACS results for Tregs from a single experiment. In the upper panels, P2 gate represents the percentages of $CD4^+CD25^+$ T cells in the splenic cells. The lower Q1-1 gates indicate the percentages of $Foxp3^+$ lymphocytes in the P2 gate. These data were obtained at 0 day, 3 days, 1 week, 2 weeks, and 3 weeks post anti-CD25 mAb administration, respectively. Six mice were used each time. **b** Effect of the anti-CD25 mAb on the Treg frequency in splenocytes after 0 day, 3 days, 1 week, 2 weeks, and 3 weeks after anti-CD25 mAb administration. Data are presented as means ± SDs from three independent experiments with 6 mice per group. $^*P < 0.05$ compared with the infected control group

Discussion

FABP was first isolated in 1972 by Ockner along with other components of intestinal cell lysates (Ockner et al. 1972). FABP in trematodes is a family of proteins with various isoforms in parenchymal and tegument cells. Its main function is to regulate the uptake and intracellular transport of fatty acids for fatty acid oxidation and triglyceride and phospholipid synthesis (Chemale et al. 2010). *Schistosoma* cannot resynthesize long-chain fatty acids and cholesterol molecules; however, FABP can be used to absorb, transport, and transform fatty acids in the cell membrane from host cells (Raina et al. 2004). There is evidence that the release of fatty acid derivatives from schistosomes plays an important role in the host immune attack in schistosomiasis. The anti-FABP vaccine may interfere with fatty acid and cholesterol uptake, thereby destroying the structural integrity of the membranes of many tissues; this could be an important determinant of its defense function. Thus, several studies indicate that FABP is an attractive vaccine candidate and a drug target against schistosomiasis (You et al. 2011). The recombinant plasmid pVAX/*Sj*-FABP can induce a 24–33% reduction in worms 6 weeks after infection (Wei et al. 2009). Nambi et al. (2005) reported that recombinant FABP do not decrease the insect load but rather decreases the size of the worms and the number of fecal eggs significantly. In this study, the worm and egg reduction rates for FABP were 30.30% and 38.90%, respectively, and these rates increased to 56.08% and 53.29%, respectively, through the combined treatment with anti-CD25 mAb and FABP. These results suggest that FABP has an anti-fecundity effect on *S. japonicum.*

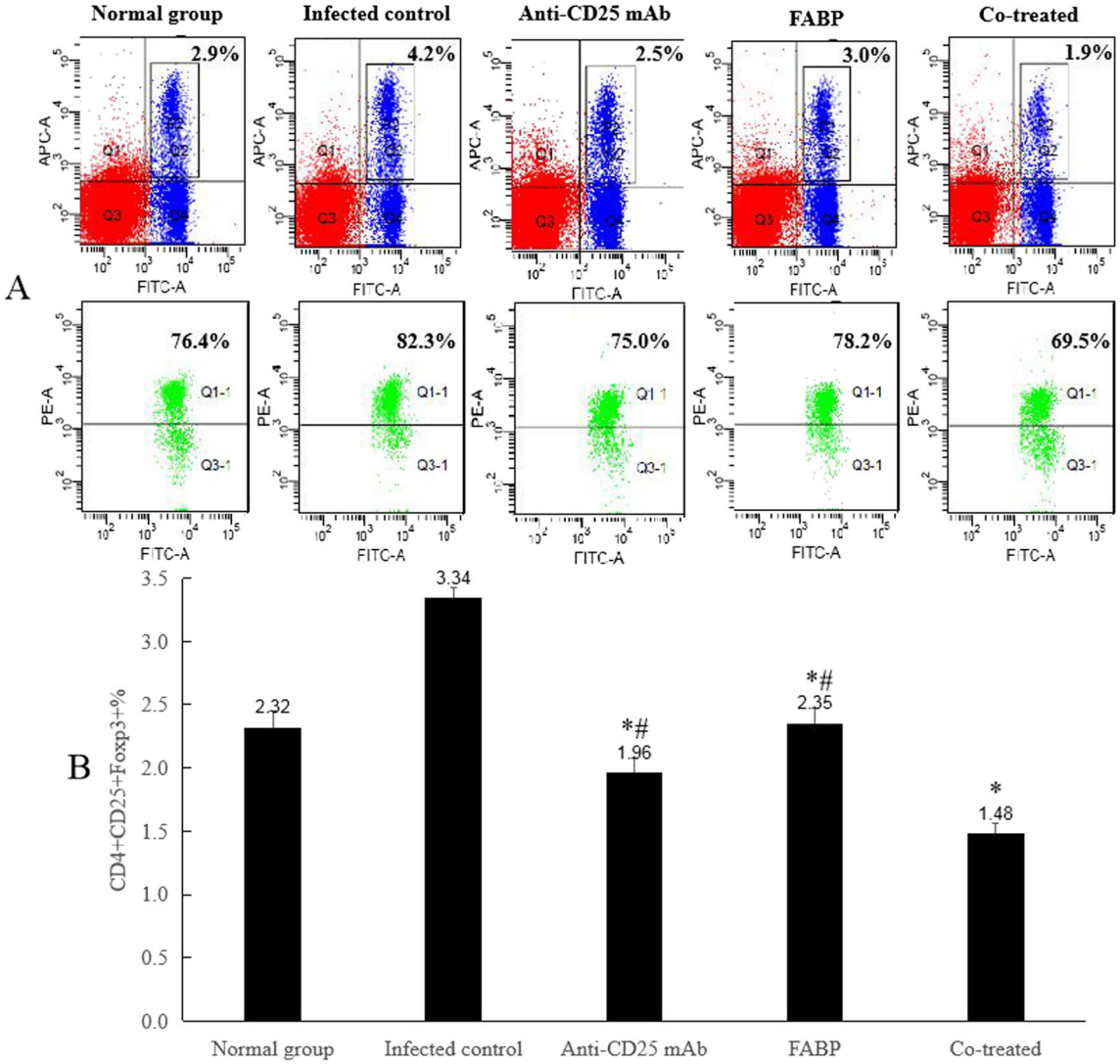

Fig. 2 **a** Representative FACS results for Tregs from a single experiment. In the upper panels, P2 gate represents the percentages of $CD4^+CD25^+$ T cells in the splenic cells. The lower Q1-1 gates indicate the percentages of $Foxp3^+$ lymphocytes in the P2 gate. These data were obtained from normal group, infected control, anti-CD25 mAb, FABP, and co-treated group, respectively. Six mice per group were used. **b** Frequencies of Tregs in total splenocytes in five groups at 5 weeks post-infection (3 weeks post anti-CD25 mAb administration). Data are from triplicate experiments with six mice per group. $*P < 0.05$ compared with the infected control group. $\#P < 0.05$ compared with the co-treated group

The mechanism of action of FABP as a vaccine is not yet clear. Recent studies indicate that the anti-parasitic effect may be related to Tregs. Tregs were first identified by Sakaguchi et al. (1995) and are a subset of $CD4^+$ T cells that also express CD25 (Suri-Payer et al. 1998). Foxp3 is a nuclear transcription factor and is a characteristic marker of Tregs (Banham et al. 2006). Immunization of $Apoe^{-/-}$ mice with the apolipoprotein B peptide vaccine aBp210 inhibits atherosclerosis. This protective effect is related to the activation of Tregs, since anti-CD25 antibody led to Treg depletion and blocked the atheroprotective effect of the vaccine (Wigren et al. 2011). Mo et al. (2018) reported that the protective efficacy of the GM-CSF prostate cancer cell vaccine may also be related to tumor-infiltrating Tregs, and norcantharidin can enhance vaccine-induced immunity via depletion of Tregs. The results of our current study demonstrated that, compared with the infected control group, the percentage of Tregs in spleen lymphocytes of FABP mice was significantly lower. The mechanism by which FABP protects against *S. japonicum* is, thus, likely related to a decrease in Tregs. Li et al. (2011) reported that the percentage of Tregs decreases significantly in the spleens of mice immunized with pEGFP-*Sj*-26GST plus cimetidine. This suggests that CIM is a potential schistosome DNA vaccine adjuvant that can enhance the protective effect of the pEGFP-*Sj*-26GST vaccine. Therefore, the strategy to reduce Tregs provides a basis for screening future vaccines. Moreover, CD25 antibodies can be used to block Tregs to enhance vaccine efficacy.

Along with Tregs, anti-CD25 monoclonal antibodies can also partially block IL-2 signaling (Wilkinson et al. 2017). In

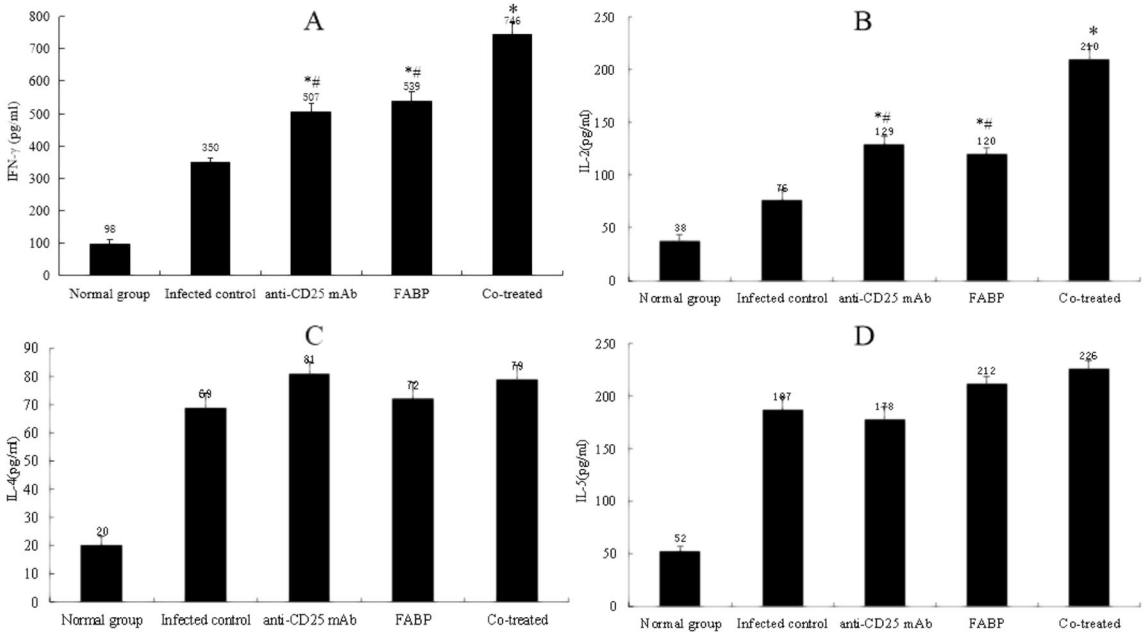

Fig. 3 Levels of IFN-γ (**a**), IL-2 (**b**), IL-4 (**c**), and IL-5 (**d**) in splenocyte culture supernatants 5 weeks post-infection. Individual mouse splenocyte suspensions were prepared from six mice per group at 5 weeks post-infection and splenocytes (5×10^6 cells/well) were cultured in RPMI-1640 supplemented with 10% FCS and 1% penicillin and streptomycin. Cultures were incubated with 5 μg/ml SEA for 72 h at 37 °C in 5% CO_2. Data are represented as means ± SD from triplicated experiments. $^{*}P < 0.05$ versus the infected control group. $^{\#}P < 0.05$ compared with the co-treated group

this study, at 2 weeks after infection (i.e., shortly after Ab injection), we observed a pronounced inhibition of Treg, i.e., more than 99% of $CD4^+CD25^+Foxp3^+$ T cells were inhibited, which might enhance the anti-schistosomula-directed immune response and thereby improve the FABP vaccine efficacy. Our results also showed that the percentage of $CD4^+CD25^+Foxp3^+$ T cells decreased at 3 and 4 weeks after infection (i.e., 1 and 2 weeks after the administration of anti-CD25 mAb). This period is characterized by susceptibility of the schistosomula and favors the efficacy of the vaccine. Hu et al. (2018) obtained similar results, showing that injection with the PC61 (anti-CD25) mAb reduces the number of Tregs by 50% in the spleen and 60% in the lung of septic mice. Combination therapy with anti-CD25 mAb and FABP was more effective in inhibiting tumor growth than FABP alone. Generally speaking, this is the first report indicating that an anti-CD25 mAb could block Tregs and improve FABP-induced immunity. Our earlier study also confirmed that the protective efficacy of the GST vaccine against *S. japonicum* is related to Treg induction, and that anti-CD25 mAb can partially block Tregs, thereby enhancing the protective efficacy of the GST vaccine (Tang et al. 2017).

According to the different cytokine types produced during the activation of mouse Th cell clones, Mosmann et al. (2005) proposed two functional subgroups, Th1 and Th2. This classification is expected to clarify the human protective mechanism and ultimately be useful for vaccine development. Accordingly, a large number of studies have focused on the Th1/Th2 balance (Bretscher 2014). Some studies have shown that IFN-γ-mediated cell death of *S. japonicum* is the main mechanism explaining 60–80% protection in attenuated cercariae-immune animals. Therefore, in schistosomiasis vaccine research, the ability of candidate molecules to induce the Th1 immune response is often considered when screening vaccine candidates. FABP induces an IgGl dominant response, with significant IgE, IgG2a, and IgG2b responses, indicating a mixed Th1/Th2 response (Smooker et al. 2001). The Th1 immune response protects against murine schistosomiasis (Fonseca et al. 2004). Native *F. gigantica* FABP is a promising vaccine candidate against *S. mansoni* infection and elicits mixed IgG(1)/IgG(2b) immune responses with IgG1 as the predominant isotype, suggesting that the protective effect of native *F. gigantica* FABP is mediated by a mixed Th1/Th2 response (Rabia Aly et al. 2012). The results of this study showed that recombinant *Sj*-FABP immunization and anti-CD25 mAb could induce host production of Th1 type cytokines, with potential synergistic effects. There was no significant difference in the Th2 type immune reaction among infected groups. It is known that Treg depletion can lead to disruption of the Th1/Th2 axis to the advantage of Th1 (Xiong et al. 2015). Thus, the protective effect of FABP and anti-CD25 mAb, when administered alone or in combination, may be achieved by the enhancement of the Th1 type immune response.

In conclusion, our study verifies that the protective mechanism of FABP may be related to a decrease in Tregs. Additionally, anti-CD25 mAb can enhance the immune protective effect by further decreasing Tregs and enhancing the Th1 type immune response.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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