

# Anti-CD25 monoclonal antibody enhances the protective efficacy of *Schistosoma japonicum* GST vaccine via inhibition of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells

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**Abstract** The effect of anti-CD25 monoclonal antibody (anti-CD25 mAb) on the protection efficacy of *Schistosoma japonicum* 26 kDa GST (glutathione-S-transferase) vaccine was evaluated. Mice were immunized with GST before infection with *S. japonicum* cercariae and then injected with anti-CD25 mAb. The worm reduction rate was promoted from 24.18% in mice with GST immunization to 47.09% in mice with GST plus anti-CD25 mAb. Compared with the control group, the percentages of splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) were significantly lower after administration of anti-CD25 mAb; meanwhile, elevated levels of IFN- $\gamma$  and IL-2 were secreted by splenocytes. These results indicate that the poor protective efficacy of the GST vaccine against *S. japonicum* results from the presence of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, while anti-CD25 mAb can partially block CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and thus enhance the protective efficacy of the GST vaccine.

**Keywords** *Schistosoma japonicum* · GST vaccine · CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>Tregs · Anti-CD25 mAb

## Introduction

Studies on vaccines against *Schistosoma japonicum*, including dead vaccine, attenuated live vaccine, and genetically

engineered and nucleic acid vaccine have often yielded disappointing results (You and McManus 2015; Tebeje et al. 2016). Glutathione-S-transferase (GST) is one of the candidate vaccines against *S. japonicum* recommended by WHO, but it usually requires not more than 40–60% protective efficacy (Bergquist et al. 2002). Previous studies showed that parasites have developed sophisticated strategies to escape from the host immune assault, including evasion of humoral and cellular immunity by antigenic variation or interference with antigen processing by cells of the innate immune system (Sacks and Sher 2002). Findings from our group suggest that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) induced by parasites have the potential to prevent complete clearance of the parasite (Tang et al. 2011). Several T cell subsets with suppressive properties have been described, and one of the best-characterized populations of murine regulatory CD4<sup>+</sup> T cells is defined by a constitutive expression of the alpha chain of the IL-2 receptor (CD25) (Sakaguchi et al. 1995). Apparent roles for CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in allowing parasite escape from host immunity have been demonstrated in various acute and chronic infection models by depleting CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs with anti-CD25 monoclonal antibody (anti-CD25 mAb) (Onyilagha et al. 2014; Tang et al. 2011). Stober et al. (2005) demonstrated that a vaccine against *Leishmania major* can induce regulatory T cells that can interfere with the efficiency of the protective immune response. Espinoza Mora et al. (2014) also reported that anti-CD25 mAb can partially block CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and enhance the protective efficacy of a vaccine against malaria. In this paper, we studied the effect of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs on the protective efficacy of the GST vaccine and demonstrated that anti-CD25 mAb enhances the protective efficacy of GST against *S. japonicum* by blocking CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in BALB/c mice.

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## Materials and methods

### Animals and parasites

BALB/c female mice, 6–8 weeks old, were obtained from Hubei Province Center for Disease Control and Prevention, China. The experiment was approved by the Committee on Animal Research of Wuchang Hospital (No. 2014-0013). The mice were divided randomly into 5 groups: normal group (uninfected), infected control group, anti-CD25 mAb group, GST group, and co-treated  $\gamma$ thyc=5 group with GST and anti-CD25 mAb. *Oncomelania* snails infected with *S. japonicum* were supplied by Jiangxi Province Institute of Parasitosis Control and Prevention, China. *S. japonicum* cercaria were shed from the snails.

### Immunization schedule and challenge infection

The mice in the GST group and co-treated group with GST and anti-CD25 mAb were primed percutaneously with 50  $\mu$ g of GST against *S. japonicum* according to Yu et al. (2006) and boosted twice with the same dosage at 2-week interval. The mice in the other three groups received an equal volume of PBS. At 2 weeks after the last immunization, all mice were percutaneously infected with 40 cercaria of *S. japonicum* except for the mice in the uninfected group. Based on own data (Tang et al. 2014), at 2 weeks post-infection, the mice in the anti-CD25 mAb group and co-treated group were injected intraperitoneally with 300  $\mu$ g of anti-CD25 mAb (eBioscience, PC61) and equal volume of PBS for mice in the other three groups, respectively (Baumgart et al. 2006). All mice were sacrificed at 5 weeks post-infection (3 weeks post anti-CD25 mAb administration). The spleens of sacrificed mice were collected for detection of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and cytokines.

### Assessment of worm and egg burdens

To assess the protective efficacy of GST vaccine, all mice in the infected groups were sacrificed and perfused from portal vein at 5 weeks post-infection according to Ruppel et al. (1990). The number of worms was counted under a dissecting microscope. Reductions in parasite burden were calculated. To determine the egg burden in the liver, mice livers were removed and 0.5 g of the large left lobe of each liver was digested at 37 °C for 3 h in 20 ml of 5% potassium hydroxide (KOH). Four 250  $\mu$ l aliquots of each sample were counted and a mean value was determined according to Cheng et al. (2008). Then, the eggs per gram liver were calculated. The remaining liver lobe was used for pathological evaluation.

### Flow cytometric analysis

For detection of the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, single-cell suspension of splenocytes at 5 weeks post-infection was prepared according to Mo et al. (2007). Cells were stained using Mouse regulatory T cell staining Kit (eBioscience) and analyzed on FACS Calibur (Becton Dickinson) with CellQuest software. The following conjugated antibodies were incubated with lymphocyte populations: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, allophycocyanin (APC)-conjugated anti-mouse CD25, and phycoerythrin (PE)-conjugated anti-mouse Foxp3 (forkhead box P3). PE-conjugated rat IgG2 $\alpha$  served as isotype control (eBioscience).

### Splenic cell culture for murine cytokines

Individual mouse splenocyte suspension was prepared from 6 mice in each group at 5 weeks post-infection, and splenocytes ( $5 \times 10^6$  cells/well) were cultured in RPMI-1640 supplemented with 10% FCS and 1% penicillin and streptomycin (all from Sigma). Cultures were incubated with or without 5  $\mu$ g/ml SEA for 72 h at 37 °C in 5% CO<sub>2</sub>. The concentrations of IFN- $\gamma$ , IL-2, IL-4, and IL-5 in cell culture supernatants were determined according to the instruction of the ELISA Kits (eBioscience). In brief, mouse IFN- $\gamma$ , IL-2, IL-4, and IL-5 were detected by biotinylated monoclonal antibodies, which were evidenced by avidin-conjugated horseradish peroxidase followed by incubation with TMB substrate. OD values at 450 nm were recorded using MK3 microplate reader.

### Histologic evaluation of granuloma formation

The remaining right liver lobe of each mouse was fixed in 10% buffered formaldehyde, embedded in paraffin, and cut into about 5- $\mu$ m sections. The sections were stained with hematoxylin and eosin (H&E). Representative H&E-stained liver sections from each animal were scanned under  $\times 200$  magnifications with a compound microscope.

### Statistical analysis

All data were expressed as mean  $\pm$  S.D. and analyzed with SPSS 17.0. Comparison among different groups was made with ANOVA. A value of  $P < 0.05$  was considered as significant.

## Results

### The effects of anti-CD25 mAb on the protection of GST vaccine in mice

To determine the protective efficacy of GST vaccine, the worm and egg burdens in 6 mice in each group were detected at 5 weeks

post-infection. The experiment was performed twice. As shown in Table 1, the worm burden in mice co-treated with GST and anti-CD25 mAb was significantly lower than those in mice treated with GST alone. Also, the egg burden in the livers of co-treated mice was clearly reduced.

### The effect of anti-CD25 mAb on the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> T cells in the spleen

Next, we addressed the question whether CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs might be linked to the low protective efficacy of GST vaccination. Since Foxp3 is a specific marker of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (Wan and Flavell 2007), we determined the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> T cells as well as CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs within the total splenocytes and the CD4<sup>+</sup> T cell subset in all mouse groups.

As shown in Figs. 1 and 2, the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells were increased after infection with *S. japonicum*. Of note, frequencies were even higher in the mice of the GST group. At 3 weeks after administration of anti-CD25 mAb (5 weeks after infection), the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in both anti-CD25 treated groups were significantly lower than those in the infected control group and returned to levels as found in uninfected mice ( $P < 0.05$ ). As shown in Fig. 3, there was no significant difference in percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> T cells among the groups.

### Cytokine production by splenocytes after vaccination and anti-CD25 mAb treatment

Vaccine immunity is known to target the schistosomula stage, for which it usually takes about 4 weeks to develop to the adult stage. In schistosome-infected mice, an initial Th1-dominated immune response switches to a Th2 profile upon the deposition of eggs by adult worms (Grzych et al. 1991). Figure 4 shows that after administration of anti-CD25 mAb, levels of the Th1 cytokines IFN- $\gamma$  (Fig. 4a) and IL-2 (Fig. 4b) were higher than in groups without anti-CD25 mAb ( $P < 0.05$ ).

There was no significant difference for the Th2 cytokines IL-4 (Fig. 4c) and IL-5 (Fig. 4d) between infected groups.

### Histologic evaluation of granuloma formation

Typical egg-induced granuloma formation was found in the infected groups, as well as cellular infiltration including lymphocytes, mononuclear cells, and eosinophil granulocytes. In addition, minimal hepatocyte necrosis in the vicinity of eggs was found. No significant differences in granuloma size and pathological consequences were found between infected groups (data not shown).

### Discussion

It is well established that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs play an important role in the immune escape of parasites (Stephen-Victor et al. 2017). There is some evidence that infection-induced CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs may potentiate the pathogens' survival by suppression of protective host immune responses (Abel et al. 2012).

In the present paper, we studied the effect of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs on the protection of the GST vaccine against *S. japonicum* in mice. The results show that GST vaccine against *S. japonicum* enhances the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the infected animals and it implies that the poor protection of GST against *S. japonicum* may be linked to the activity of CD4<sup>+</sup>CD25<sup>+</sup> Tregs. It has been shown that co-treatment with TLR7 ligands (Wang et al. 2013) or cimitidine (Li et al. 2011) improved the protective efficacy of a GST DNA vaccine against *S. japonicum* infection which was associated with a reduction of Treg frequencies. Toka et al. (2004) demonstrated that anti-CD25 mAb can partially block CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs to enhance the immune response of a vaccine against *herpes simplex virus* and to clear the virus from the host. To determine the effect of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs on the protection of GST vaccine, we blocked CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs with anti-CD25 mAb at 2 weeks

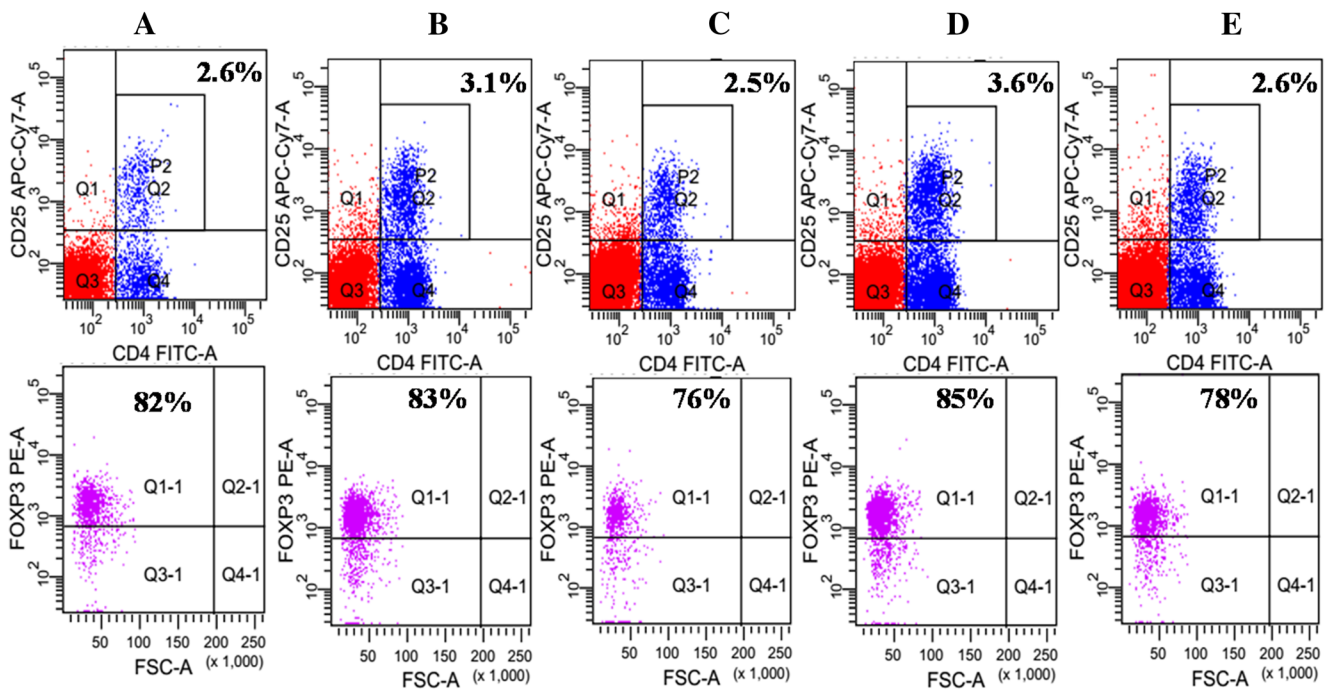
**Table 1** The effect of anti-CD25 mAb on the protective efficacy of GST against *S. japonicum* in BALB/c mice

Group	Worm burden	Worm reduction rate (%)	Average number of eggs per gram liver tissue ( $\times 10^3$ )	Liver egg reduction rate (%)
Infected control	28.16 $\pm$ 3.02		22.6 $\pm$ 8.50	
Anti-CD25 mAb	21.80 $\pm$ 3.32	22.58 <sup>a</sup>	18.8 $\pm$ 2.8	16.81
GST	21.35 $\pm$ 5.68	24.18 <sup>a</sup>	14.9 $\pm$ 6.7	34.07 <sup>a</sup>
GST + anti-CD25 mAb	14.90 $\pm$ 2.28	47.09 <sup>b</sup>	11.3 $\pm$ 6.2	50.00 <sup>a</sup>

Data are presented as mean  $\pm$  S.D. from 2 independent combined experiments (6 mice in each group)

<sup>a</sup> $P < 0.05$ , versus infected control group

<sup>b</sup> $P < 0.05$ , versus GST group



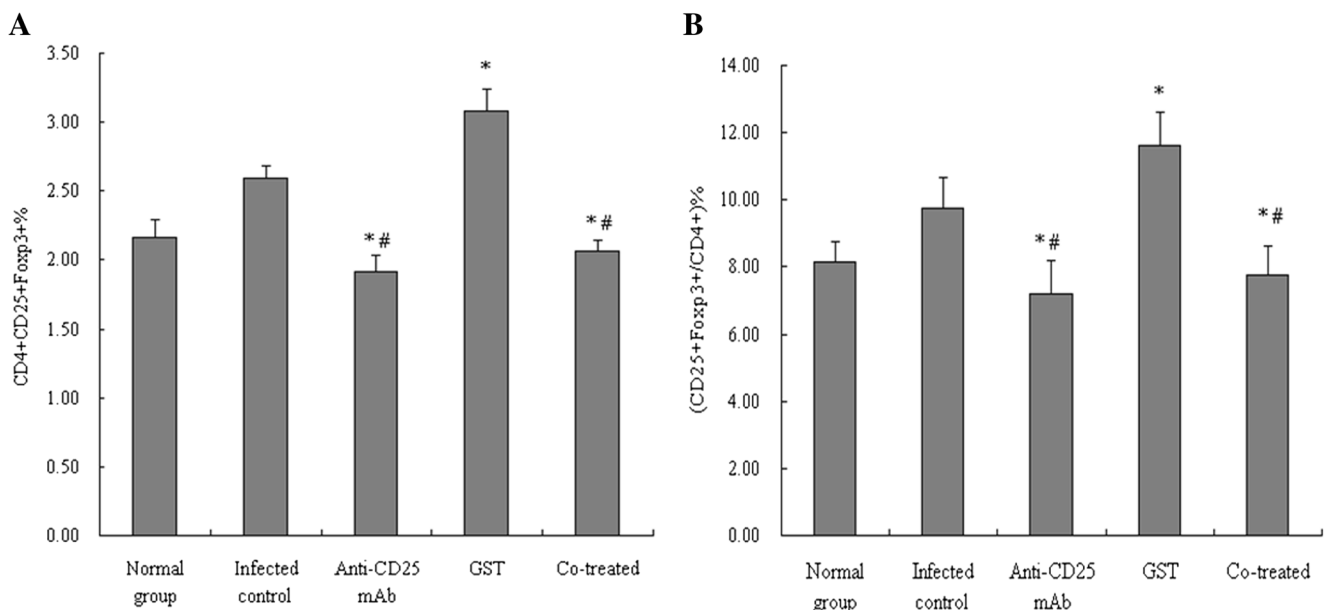
**Fig. 1** Representative FACS result of  $CD4^+CD25^+Foxp3^+$  Tregs and  $CD4^+CD25^+Foxp3^-$  T cells from on experiment. Uninfected control (a), infected control (b), anti-CD25 mAb (c), GST (d), and co-treated group (e). Upper panels, P2 gate denotes the percentages of

$CD4^+CD25^+$  T cells in splenocytes. Q1-1 gate in the lower panel indicates the percentages of  $Foxp3^+$  lymphocytes in P2 gate. These figures are from one experiment at 5 weeks post-infection (i.e., 3 weeks post anti-CD25 mAb)

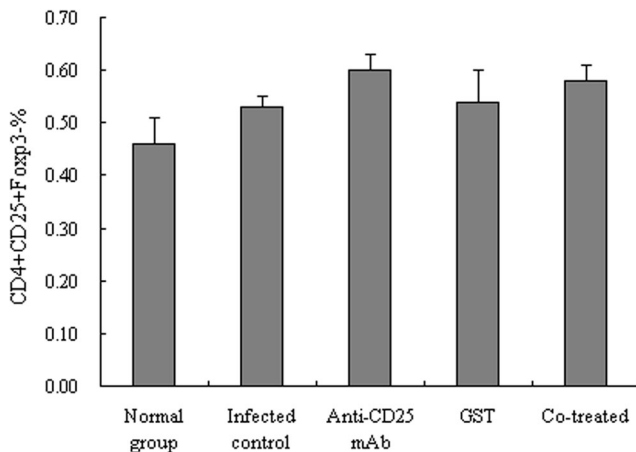
post-infection with *S. japonicum*. This considerably reduced the frequency of  $CD4^+CD25^+Foxp3^+$  Tregs in splenocytes and at the same time increased the protection of GST vaccine.

Although a Th1-type immune response is important for the host to clear *S. japonicum* (Tang et al., 2014), infection causes a parasite-induced immunological switch from an early Th1

response to a Th2-response after schistosome maturation and the onset of egg production. Our data show that the administration of anti-CD25 mAb results in increased levels of the Th1 cytokines  $IFN-\gamma$  and IL-2 secreted by splenocytes. This indicates that anti-CD25 treatment enables the host to reduce *S. japonicum* burden through reduction of



**Fig. 2** The effect of anti-CD25 mAb on frequencies of  $CD4^+CD25^+Foxp3^+$  T cells within total splenocytes (a) and the  $CD4^+$  T cell subset (b). Data are presented as mean  $\pm$  S.D. for triplicate experiments with 6 mice per group. \* $P < 0.05$ , versus infected control group. # $P < 0.05$ , versus GST group



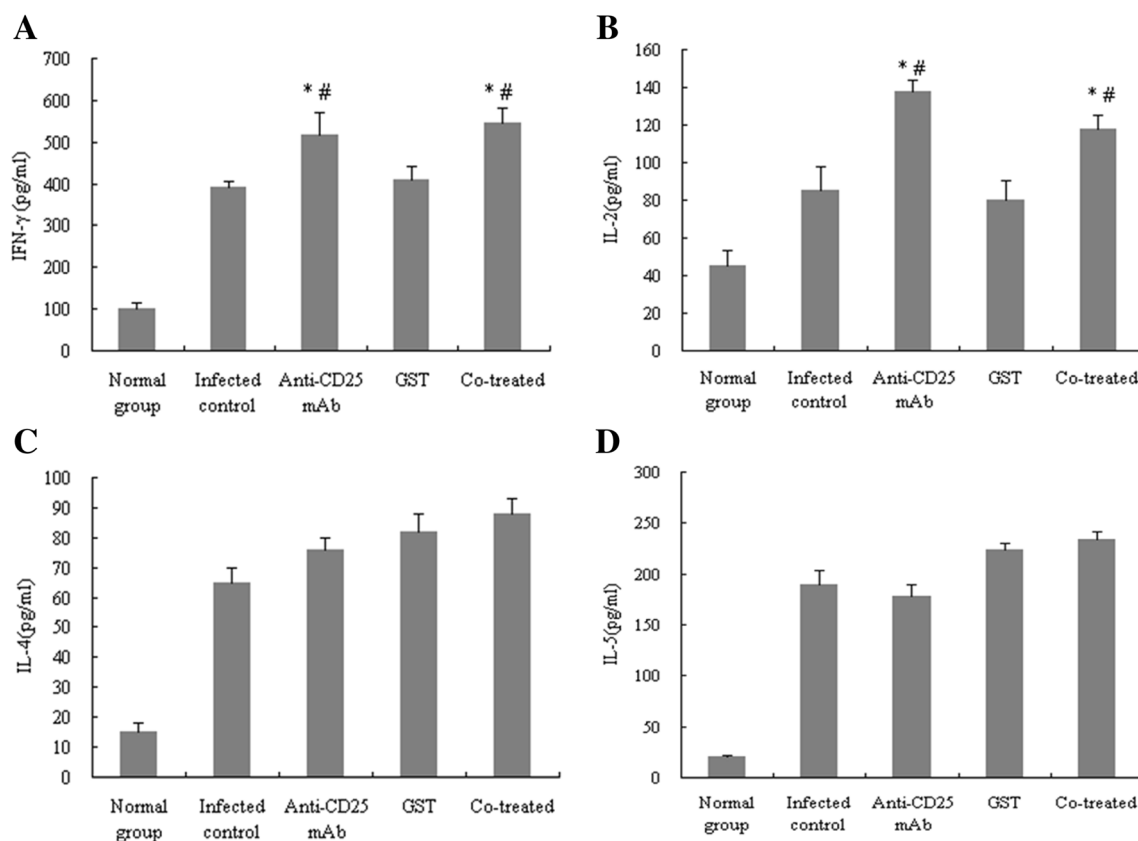
**Fig. 3** The effect of anti-CD25 mAb on frequencies of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>-</sup> T cells within splenocytes. Data are presented as mean  $\pm$  S.D. for triplicate experiments with 6 mice per group

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and enhancement of Th1 responses.

It has been shown that protective immunity targets schistosomula before they develop into adult worms. The majority of schistosomula are killed within the first few days of

infection until 2–4 weeks thereafter (Oswald et al. 1994). A few days after administration of anti-CD25 mAb, the percentages of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in groups with anti-CD25 mAb dramatically dropped down; 3 weeks thereafter, the percentages slowly rose but still significantly lower than those in the groups without anti-CD25 mAb ( $P < 0.05$ , unpublished). We targeted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs by treating mice with anti-CD25 mAb at 2 weeks post-infection, which means within the critical period of schistosomula susceptibility to vaccine immunity. This further supports the idea of improved vaccine efficacy by attacking CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs.

Also, anti-CTLA-4 was described to enhance parasite clearance by neutralizing CTLA-4 signaling (Taylor et al. 2007). However, anti-CTLA-4 treatment resulted in immune-mediated adverse effects and enhanced pathological reactions (Sheik Ali et al. 2015). Therefore, this approach seems to be unsuitable to enhance vaccine efficacy. On the contrary, in our study, anti-CD25 mAb improved vaccine efficacy without aggravating the pathological consequences caused by eggs. This is also reflected by the presence of enhanced Th1-type immune responses upon anti-CD25 mAb treatment, while pathology caused by *S. japonicum* eggs is generally related to Th2-type



**Fig. 4** The levels of IFN- $\gamma$  (a), IL-2 (b), IL-4 (c), and IL-5 (d) in splenocytes cultural supernatant 5 weeks post-infection. Individual mouse splenocyte suspension was prepared from 6 mice in each group at 5 weeks post-infection and splenocytes ( $5 \times 10^6$  cells/well) were cultured in RPMI-1640 supplemented containing 10% FCS and 1%

penicillin and streptomycin. Cultures were incubated with 5  $\mu$ g/ml SEA for 72 h at 37  $^{\circ}$ C in 5% CO<sub>2</sub>. Data represent mean  $\pm$  S.D. from triplicate experiments. \* $P < 0.05$ , versus infected control group. # $P < 0.05$ , versus GST group



immune responses. Therefore, anti-CD25 mAb has the potential to be applied as enhancer of future schistosomiasis vaccine immunity.

In conclusion, we demonstrate that the poor protective efficacy of GST vaccine against *S. japonicum* is related to the presence of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs, which potentially favor the immune evasion of parasites. Anti-CD25 mAb significantly enhanced the protection potential of the GST vaccine by reducing CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs and enhancement of Th1 type immune responses.

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**Compliance with ethical standards** The experiment was approved by the Committee on Animal Research of Wuchang Hospital (No. 2014-0013).

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

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