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Effects of *Microtus fortis* lymphocytes on *Schistosoma japonicum* in a bone marrow transplantation model

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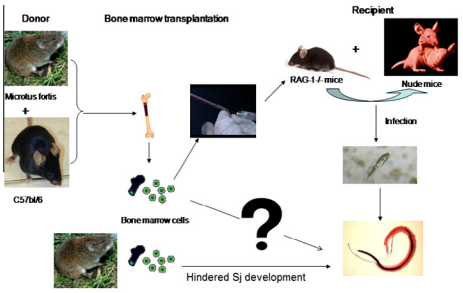
HIGHLIGHTS

- We established BMT models from *M. fortis* and C57BL/6 mice to immunodeficient mice.
- BMT models were established successfully.
- The *M. fortis* lymphocytes did not appear to affect the *S. japonicum* worm burden.
- The *M. fortis* lymphocytes led to worm shortening and reduction in spawning.

GRAPHICAL ABSTRACT



Different growth and development of *shistosoma japonica* in different transplantation models

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ABSTRACT

Microtus fortis is a non-permissive host for *Schistosoma japonicum*. While *M. fortis* lymphocytes are known to provide natural resistance against *S. japonicum*, the specific mechanism remains unclear. A bone marrow transplantation (BMT) model was established using immunodeficient mice, either nude (experiment 1) or V(D)J recombination activation gene deficient mice (RAG-$1^{-/-}$) (experiment 2) as recipients and *M. fortis* or C57BL/6 mice as donors. The growth and development of *S. japonicum* were evaluated in each group to assess the role of *M. fortis* lymphocytes in the response to infection. Lymphocyte ratios and *S. japonicum*-specific antibody production in transplanted groups increased significantly compared to those in non-transplanted group. Spleen indices and density of splenic lymphocytes in transplanted RAG-$1^{-/-}$ mice were higher than those in non-transplanted RAG-$1^{-/-}$ mice. No difference in the worm burden was observed among group A (transplants derived from *M. fortis*), B (transplants derived from C57BL/6 mouse) and C (non-transplanted mice), although worms in group A were shorter than those in other groups, except non-transplanted RAG-$1^{-/-}$ mice. Reproductive systems of worms in mice (nude or RAG-$1^{-/-}$) transplanted from *M. fortis* were not as mature as those in mice (nude or RAG-$1^{-/-}$) transplanted from C57BL/6 mouse and non-transplanted nude mice, but they were more mature than worms in non-transplanted RAG-$1^{-/-}$ mice. Therefore, the transplantation model using nude and RAG-$1^{-/-}$ mice was successfully established. The *M. fortis* lymphocytes did not appear to affect the *S. japonicum* worm burden, but they led to schistosome shortening and a significant reduction in parasite spawning. Thus, *M. fortis* cellular and humoral immunity provides a defense against schistosomes by negatively impacting the parasite growth and reproductive development.

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1. Introduction

Microtus fortis is a non-permissive host for *Schistosome japonicum* with a genetic predisposition for resistance against schistosomiasis (He et al., 1995). The larvae arrive at the *M. fortis* lung within 2 days and at the liver within 4 days of schistosome infection. At 11 days post-infection, the migration and development of the schistosomulum in *M. fortis* coincide with those in mice. By the 12th day post-infection, the schistosomulum growth is inhibited, and the worms gradually shrink and ultimately die by 3 weeks post-infection (Lu, 1982). C57BL/6 mice are permissive hosts of the parasite, and schistosomes can cause hepatic fibrosis as they mature in these mice (Hou et al., 2012). Accordingly, a comparison of the schistosome development in *M. fortis* and C57BL/6 mice may be helpful for understanding the mechanisms behind the *M. fortis* resistance to the parasites.

Previous studies have implicated both immunological and non-immunological factors in the resistance against schistosome infection in *M. fortis*. The immunological factors include resistance-related cells (e.g., eosinophils, neutrophils and macrophages), complement, interleukin (IL)-4 and immunoglobulin-G3 (IgG3) (Hu and Cao, 2010). The non-immunological factors include nitric oxide, albumin, E77, KPNA2 and HSP90 (Hu and Cao, 2010; Cheng et al., 2011; Gong et al., 2010). However, the specific mechanism and whether it involves the *M. fortis* immune system remain unclear.

Bone marrow transplantation (BMT) is an important method for the treatment of hematopoietic dysfunction, immune deficiency, hematological cancer and other cancers. Upon transplantation into severe combined immunodeficiency (SCID) mice, mouse bone marrow mononuclear cells (BMMCs) can grow and differentiate, thus rebuilding immune function in these mice (Liang and Spear, 2008).

In our current study, BMMCs from *M. fortis* and C57BL/6 mice were transplanted into nude and RAG-$1^{-/-}$ mice, restoring to a certain extent the immune function of these immunodeficient mice. After infection with schistosomal cercariae, growth and development of the schistosomes in these transplanted mice were observed and compared to explore the role of *M. fortis* lymphocytes in the host immune defense during schistosomiasis.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide obtained from the Laboratory Animal Welfare & Ethics Committee (LAWEC) of China. No specific permission was required from Hunan provincial authorities, since the study did not involve endangered or protected species. The protocol was approved by the Committee on LAWEC of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (approval ID: IPD 2009-4). Surgeries were performed using sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Animals and cercariae

M. fortis were live captured in the Hunan Dongtin Lakes region. Small mammals were transported to Shanghai and reared in independent ventilated cages. Female *M. fortis* were selected randomly from sub-generations of wild animals for the experiments. C57BL/6 and nude mice (7–8 weeks of age) were purchased from the Experimental Animal Department of the Chinese Academy of Sciences. RAG-$1^{-/-}$ mice were purchased from the Model Animal Research Center of Nanjing University. The genetic background of nude mice is BALB/c, and that of RAG-$1^{-/-}$ mice is C57BL/6. The animals were infected cutaneously (see below) with a mainland strain of *S. japonicum* cercariae. Infected *Oncomelanias hupensis* snails were provided by the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. The infected snails were placed in dechlorinated water under artificial light to induce cercarial shedding prior to animal infections.

2.3. Isolation of BMMCs

The *M. fortis* and C57BL/6 mice were anesthetized with an intraperitoneal injection of sodium pentorbital (100 mg/kg), sacrificed and immersed in 75% ethanol for 5 min. The femoral and tibia bones were aseptically excised from the donor (*M. fortis* or C57BL/6) and washed in sterile RPMI1640 medium (Invitrogen, Carlsbad, CA). After resection, the bone marrow in the medullary cavity was flushed with heparin-containing RPMI1640 (50 U/mL) using a 23-G needle and a 1-mL syringe. After lysing the red blood cells, the bone marrow cells were brought to a single cell suspension at 5.0×10^7 cells/mL in injectable 0.9% sodium chloride. The viability of BMMCs was greater than 95% as measured by trypan blue exclusion. A total of 1×10^7 BMMCs were injected via the tail vein into each recipient mouse.

2.4. Establishment of the transplantation model and animal infections

Healthy nude and RAG-$1^{-/-}$ mice were used as recipients, while *M. fortis* and C57BL/6 mice were used as donors. Five days prior to transplantation, gentamicin sulfate (320 mg/L) and erythromycin (250 mg/L) were added to the sterile drinking water of the recipient mice. The nude and RAG-$1^{-/-}$ mice (excluding the control group) were lethally irradiated with a single dose of 400 cGy using a 137 Cs irradiator (Institute of Immunology, Shanghai Jiao Tong University School of Medicine). Four hours after irradiation, the recipient mice were intravenously injected with 1×10^7 unfractionated BMMCs isolated from wild-type donor mice. In the first animal experiment, animals were divided into five groups, including group A (8 nude mice transplanted from *M. fortis*), group B (8 nude mice transplanted from C57BL/6 mouse), group C (8 non-transplanted nude mice), group D (8 normal C57BL/6 mice as control) and group E (8 normal *M. fortis* as control). In the second experiment, RAG-$1^{-/-}$ mice were divided into three groups, including group A (8 RAG-$1^{-/-}$ mice transplanted from *M. fortis*), in group B (8 RAG-$1^{-/-}$ mice transplanted from C57BL/6 mouse) and group C (8 non-transplanted RAG-$1^{-/-}$ mice). The non-transplanted control group was intravenously injected with 0.2 mL of saline. After 6.5 weeks, each recipient was infected with 40 ± 2 cercaria by abdominal skin exposure. All of the animals were sacrificed at 6 weeks post-infection.

2.5. Antibody detection by ELISA

All of the experimental animals were anesthetized and sacrificed at 6 weeks post-infection. Sera were collected from animals in all groups at 6 weeks post-transplantation and stored at −20 °C until further testing.

An enzyme-linked immunosorbent assay (ELISA) was used to determine the serum level of *Schistosoma* worm antigen (SWA)-specific antibodies in the nude and RAG-$1^{-/-}$ mice. Polystyrene microtiter plates were coated with SWA (10 ng/mL) diluted in 0.05 M carbonate buffer (pH 9.6) and maintained at 4 °C overnight. The 96-well plates were blocked with 1% BSA in 0.15 M phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween-20 (PBS-T). The serum samples were diluted at 1:100 in PBS-T. The binding of SWA-specific IgG was detected with goat anti-mouse peroxidase-conjugated IgG (Bio-Rad, Hercules, CA) diluted at 1:10,000 in

PBS-T. Between each step, the plates were rinsed five times with PBS-T, and 3,3′,5,5′-tetramethylbenzidine (TMB) was used as the substrate. Optical densities (OD) were measured in an automated plate reader (MULTISKAN) at a wavelength of 450 nm. An OD value >2.1 times the negative control was considered to be positive.

2.6. Flow cytometry analysis

To determine the degree of immunological reconstitution following BMT, peripheral blood (PB) cells were analyzed prior to the BMT, 6.5 weeks after the BMT, and 3 and 6 weeks post-infection. The lymphocyte composition was analyzed by fluorescence-activated cell sorting (FACS). Heparinized PB cells (20 μL) were resuspended in RPMI1640 containing 10% fetal bovine serum (FBS). The cells were incubated for 30 min with the following fluorochrome-conjugated antibodies: anti-mouse CD3-PE, anti-mouse CD4-FITC, anti-mouse CD8-PE-Cy5, anti-mouse B220-APC, anti-mouse IgM-PE and anti-mouse IgD-FITC (Biolegend, San Diego, CA). After treatment with a red blood cell (RBC) lysing solution (Beyotime Co., HaiMen, China) to eliminate RBCs, the labeled cells were analyzed using an FACS Calibur (BD Biosciences, New Jersey, USA) and CellQuest 1.22 software (Becton Dickinson, Franklin Lakes, NJ).

2.7. Infection parameters

Animals were weighed and sacrificed under anesthesia 42 days post-infection. Spleens were weighed immediately after excision, and spleen indices were calculated according to the following formula: spleen index (mg/g) = (weight of spleen)/body weight. The adult worms were recovered by perfusion of the mesenteric and hepatic portal veins, and the worm totals were counted. After weighing each liver, the right lobe was fixed in 10% formalin. The remaining liver tissue was weighed and digested with 8% KOH (20–40 mL) at 37 °C for 3 h in a shaking incubator at 250 rpm. The number of eggs/liver was determined in triplicate by microscopic examination of 100 μL aliquots. Worm and egg burdens were calculated as previously described (Hu et al., 2012).

Adult worms were recovered from recipient mice 6 weeks post-cercarial infection. The worms were washed twice with physiological saline solution and fixed overnight at room temperature in FAA (5% acetic acid, 5% formaldehyde and 63% ethanol). After fixation, the worms were stained with hydrochloric carmine, cleared with 0.5% hydrochloric ethanol and dehydrated with 2-h incubations in serial grades of ethanol (70%, 90% and 100%). Following a final incubation in xylene, the worms were preserved in Canada balsam. The worms were then analyzed by laser scanning confocal microscopy LSM510 (Zeiss, Jena, Germany) using a 543 He/Ne laser with an LP 560 filter. Worm sizes were determined using a stereo microscope SZX12 (Olympus, Tokyo Shinjuku, Japan) and Image Pro Plus-version 3.0 software (Media Cybernetics, Bethesda, MD).

2.8. Histopathological analyses

Hepatic tissues from all of the animals were fixed in 4% buffered formaldehyde solution and embedded in paraffin. Five micrometer sections were then stained with hematoxylin and eosin (H&E) and examined by bright-field microscopy. For the evaluation of granuloma sizes, 10–15 lesions/specimen were measured. Mean values obtained for each group were used for statistical analyses. The area surrounding granulomas was measured using Image Pro Plus, version 3.0.

Histopathological analysis was performed to determine the density of lymphocytes after BMT. Splenic tissues from recipient mice were embedded in paraffin and cut into 5-μm thick sections. After deparaffinization, the tissue sections underwent antigen retrieval by exposure to high pressure for 10 min. The sections were then incubated with 3% H_2O_2 for 10 min to block endogenous peroxidase. The sections were then incubated with 10% normal goat serum for 10 min to block non-specific binding. Rat anti-mouse CD3 and IgM antibodies were diluted 1:10 in PBS/1% BSA and incubated at 4 °C overnight. After five washes, a biotin-labeled secondary antibody diluted 1:50 in PBS/1% BSA was added, and the slides were incubated for 1 h. After an additional five washes, horseradish peroxidase (HRP)-labeled streptavidin was added, and the slides were incubated for another 30 min. Diaminobenzidine (DAB) was used as a substrate to provide color, and the slides were counter-stained with hematoxylin. Finally, the sections were mounted and imaged.

2.9. Statistical analysis

Significant differences were evaluated using analysis of variance (ANOVA) and Student's *t*-test with SAS software (Cary, NC). A P-value < 0.05 was considered to be statistically significant. All data were expressed as the mean ± standard deviation (SD).

3. Results

Immunodeficient mice were treated with a single dose of unfractionated bone marrow that was injected intravenously via the tail vein. After 6.5 weeks, the mice were infected with cercariae by abdominal skin exposure. The recipient mice were analyzed at 6.5, 9.5 and 12.5 weeks post-BMT. Mice that received transplants showed signs of reconstitution of both humoral and cellular immune functions.

3.1. Analysis of immune reconstitution

At 6.5 weeks post-transplantation, proportions of total lymphocytes, $CD3^+$ T-cells, $B220^+$ B-cells and $CD3^+CD8^+$ T-cells in group B, were increased significantly in the recipient nude and RAG-$1^{-/-}$ mice. In group A, ratios of $B220^+$ B-cells and $CD3^+CD8^+$ T-cells were slightly increased, and the proportions of the remaining lymphocytes were unchanged. This latter finding may have been due to the inability of anti-mouse antibodies to recognize lymphocytes derived from *M. fortis*. Overall, these results showed that the BMT model was successfully established in the recipient mice. On early stages (0–3 weeks post-infection), $CD3^+CD8^+$ T-cells and mature B-cells lymphocytes were slightly increased in nude and RAG-$1^{-/-}$ transplanted groups. In the later stages (3–6 weeks post-infection), $CD3^+CD8^+$ T-cells and mature B-cells lymphocytes sustained a certain degree of damage, and the lymphocyte ratios decreased (Fig. 1A and B).

Levels of *S. japonicum*-specific antibodies in group B (RAG-$1^{-/-}$ mice transplanted from C57BL/6 mouse) were increased significantly by 12.5 weeks post-transplantation. By contrast, group A did not display a significant increase in antibodies to the parasite. This result showed that the humoral immune response of immunodeficient mice (group B) recovered following transplantation. The impact of the BMT using cells derived from C57BL/6 mice was improved over cells derived from *M. fortis* (Fig. 2A and B).

In the experiment 1, spleen indices of transplanted animals (groups A and B) showed significant differences compared to the nude control, C57BL/6 control and *M. fortis* control groups. At 12.5 weeks post-transplantation, the number of splenic lymphocytes in mice that received the BMT from *M. fortis* was 47.39% of the control *M. fortis*, and that in mice that received the BMT from C57BL/6 mice was 63.06% of the control C57Bl/6 mice. In the experiment 2, spleen indices of mice in transplanted groups were higher than that in the RAG-$1^{-/-}$ control group. The number of splenic lymphocytes in the transplanted animals (groups A and B) were significantly higher than

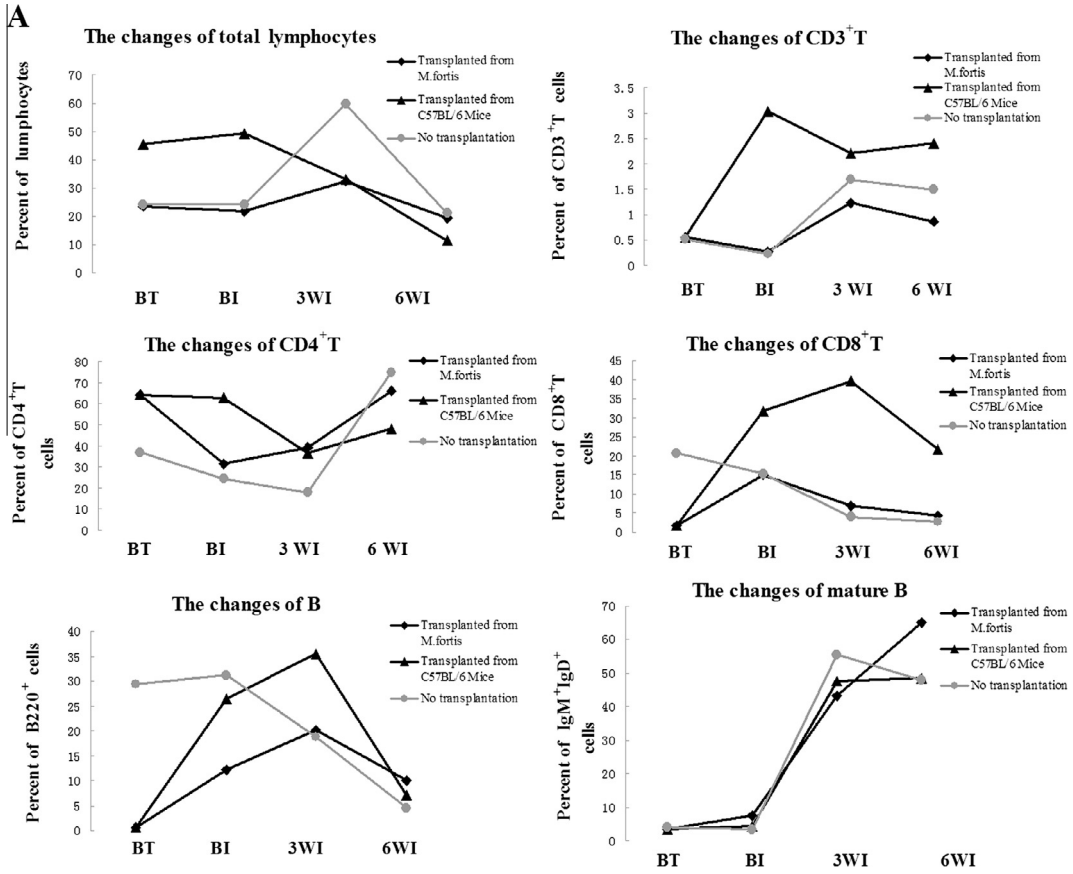

Fig. 1. Temporal changes in ratios of T and B lymphocytes of BMT recipient mice. PB cells were analyzed prior to the BMT, 6.5 weeks after the BMT, and 3 and 6 weeks post-infection. At 6.5 weeks post-transplantation, ratios of total lymphocytes, $CD3^+$ T, $B220^+$ B and $CD3^+CD8^+$ T cells were increased in the recipient nude mice (A) and RAG-$1^{-/-}$ mice (B). At 3 weeks post-infection, ratios of $CD3^+CD8^+$ T and mature B lymphocytes were slightly increased in the transplanted groups but then decreased over the following 3 weeks. Note: BT, before transplantation; BI, before infection; 3WI, 3 weeks after infection; 6 WI, 6 weeks after infection. BMT *M. fortis*, BMT from *M. fortis*; BMT mice, BMT from mice; NBMT, no BMT.

in the controls. The results showed that, at 12.5 weeks after BMT, lymphocytes in transplanted mice (group A and B) had proliferated significantly but did not recover completely due to the influence of whole body irradiation (Table 1).

3.2. Analysis of schistosomal growth and development

Worm and egg development were assessed in the different groups of mice. In the experiment 1, worm burdens between each group were not significantly different. Conversely, egg burdens (egg production rate per gram of liver, EPG) in group A (BMT from *M. fortis*) were significantly lower than in the other groups. In the experiment 2, worm burdens between each RAG-$1^{-/-}$ recipient group also were not significantly different. The egg production rate per female worm (EPF) and the EPG in group B were higher than that in group C. The reason may be that group C contained immunodeficient mice (RAG-$1^{-/-}$ mice), while group B contained immune reconstituted mice. Immunodeficiency may not be conducive to the growth of *S. japonicum* (Hu et al., 2012). However, no differences on EPF and EPG were observed between groups A and C (Table 2).

Worms were isolated from the infected nude and RAG-$1^{-/-}$ mice 42 days post-infection. Worm lengths were measured and compared by Axioplan 2 imaging. In the experiment 1, female and male worms collected from group A were shorter than those from group B, and male worms collected from group A were shorter than those from nude and C57BL/6 controls. No significant difference was detected among group B, group C and the C57BL/6 control group (Fig. 3A). In the experiment 2, female and male worms collected from group B were longer than those in the group C. Female worms collected from group A were shorter than those in group B (Fig. 3B).

Worm development and reproduction in the nude and RAG-$1^{-/-}$ mice were compared by laser scanning confocal microscopy. In the experiment 1, there were significantly fewer oocytes and spermatocytes in worms from group A (BMT from *M. fortis*) than in other groups, and contours of ovaries and testes were unclear. Conversely, numbers of oocytes and spermatocytes were unchanged between worms from group B (BMT from mice) and control mice, and contours of ovaries and testes were clear (Fig. 4A). In the experiment 2, oocytes and spermatocytes were fewer in worms from group A than from group B, although more than those from the RAG-$1^{-/-}$ control group (group C). Outlines of worm testes and ovaries were more clear in the transplanted groups than in the control group. Overall, reproductive systems of the worms in group B were more well-developed than those in group A (Fig. 4B).

In these two experiments, compared to those in recipients of BMT from C57BL/6 mice, worms collected from mice receiving the BMT from *M. fortis* were smaller and less mature. In RAG-$1^{-/-}$ mice that contained only immature lymphocytes, the *S. japonicum* worms were stunted. While a small number of lymphocytes persisted in the nude mice, the growth and development of the worms were equivalent to those in wild-type C57BL/6 mice.

3.3. Pathological changes in the transplanted model

Liver sections were H&E stained and histologically examined to assess granuloma sizes 42 days post-infection. In the experiment 1,

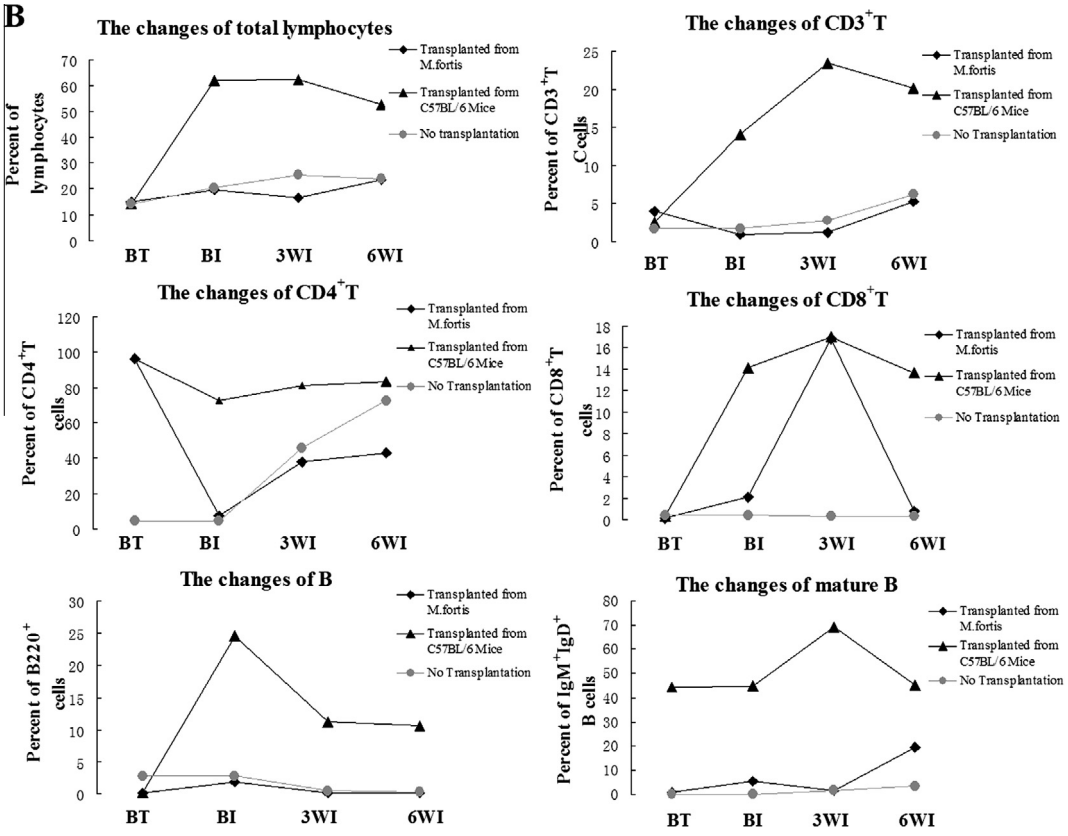

Fig. 1 (*continued*)

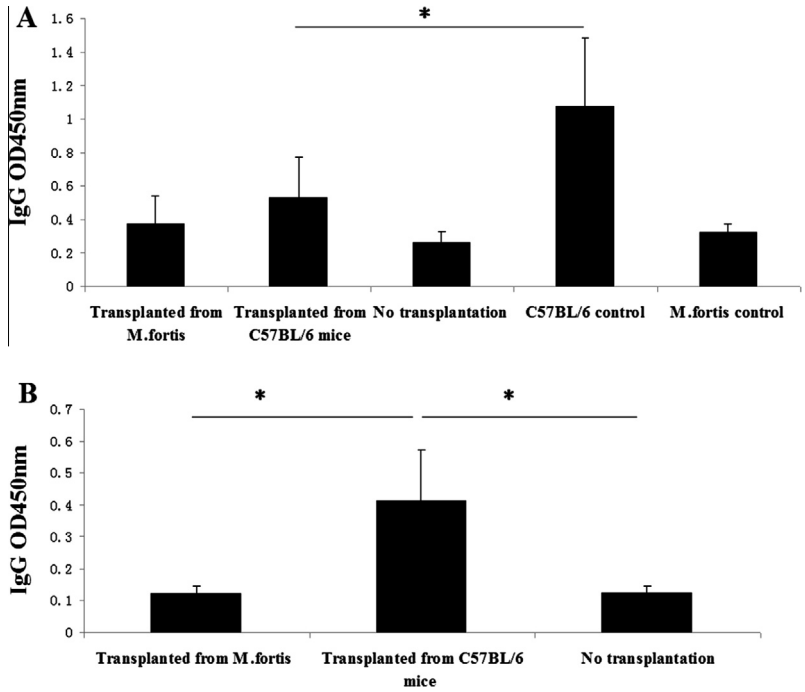

Fig. 2. *S. japonicum*-specific antibodies in sera of BMT recipient mice. Sera were collected from animals in all groups at 6 weeks post-transplantation. ELISA was used to determine levels of serum antibodies to the parasite in nude (A) and RAG-$1^{-/-}$ mice (B). At 6.5 weeks after transplantation, the *S. japonicum*-specific antibody level of group B was significantly higher than that of group C but was still lower than that of the C57BL/6 control group. This antibody level also slightly increased in group A (A), but it was lower than that of group B. $^{*}P < 0.05$.

no significant differences in granuloma sizes were observed between the transplanted and non-transplanted groups. However, in the experiment 2, granulomas of the BMT groups were larger than those of the controls, and granulomas from the *M. fortis*-derived BMT group were significantly larger than those from the mouse-derived BMT group (Fig. 5A and B).

Table 1
Spleen indices detection in transplanted models.

Experiment	Groups	Body weight	Spleen weight	Index of spleen	The total of lymphocytes in spleen ($\times 10^6$)
Experiment 1	A. Nude mice (transplanted from *M. fortis*)	25.61 ± 2.49	0.19 ± 0.02	0.76 ± 0.09[a,b,c,d]	7.36 ± 1.53[a,c]
	B. Nude mice (transplanted from C57BL/6 mice)	25.76 ± 2.29	0.24 ± 0.02	0.93 ± 0.05[c,d]	71.47 ± 25.25
	C. Non-transplanted nude mice	26.33 ± 1.43	0.40 ± 0.09	1.53 ± 0.37[c,d]	105.87 ± 61.85
	D. Normal C57BL/6 mice	23.15 ± 1.70	0.60 ± 0.04	2.60 ± 0.30[d]	113.33 ± 61.72
	E. Normal *M. fortis*	73.79 ± 7.22	0.14 ± 0.02	0.19 ± 0.04	15.53 ± 4.02
Experiment 2	A. RAG-1$^{-/-}$ mice (transplanted from *M. fortis*)	25.53 ± 4.14	0.10 ± 0.05	0.39 ± 0.21	1.30 ± 0.88[b]
	B. RAG-1$^{-/-}$ mice (transplanted from C57BL/6 mouse)	23.91 ± 3.06	0.21 ± 0.11	0.95 ± 0.64	52.60 ± 43.69[b]
	C. Non-transplanted RAG-1$^{-/-}$ mice	24.66 ± 0.59	0.06 ± 0.02	0.23 ± 0.07	0.13 ± 0.09

Note: a, $p < 0.05$, compared to group B; b, $p < 0.05$, compared to group C; c, $p < 0.05$, compared to group D; d, $p < 0.05$, compared to group E.

The density of splenic lymphocytes in the transplanted mice was detected by immunohistochemistry in the experiment 2. Numbers of T and B lymphocytes were significantly increased in the transplanted groups (A and B). Moreover, the proportion of lymphocytes increased more in the mouse-derived transplant group than in the *M. fortis*-derived transplant group (Fig. 6).

4. Discussion

Schistosomiasis is a parasitic disease of great public health importance. It is endemic in 76 countries and territories in tropical and subtropical regions. Worldwide, nearly 800 million individuals are at risk, with approximately 200 million people infected, and over half of these have varying degrees of morbidity (Lustigman et al., 2012). Schistosomes employ a variety of immune evasion mechanisms throughout their long-term interaction with the host. The lack of understanding of these mechanisms is a major impediment for vaccine research. *M. fortis*, belonging to the Rodentia family, is widely distributed in the endemic China Dongting Lake area (Lu, 1982). *M. fortis* was identified as a non-permissive host with natural resistance to *S. japonicum*. Thus, illuminating the resistance mechanisms against *S. japonicum* may be helpful in screening for new schistosomal targets to reduce its spread and minimize the disease burden.

A large number of studies have shown that the natural resistance by *M. fortis* against *S. japonicum* is related to immune factors. Macrophages and eosinophils from *M. fortis* were shown to be capable of adhering to schistosomula, which then become surrounded by *M. fortis* macrophages, eosinophils and neutrophils. This phenomenon suggests that *M. fortis* effector cells can directly kill schistosomula (Wang et al., 2002). Furthermore, the transfer of *M. fortis* serum into Kunming mice were found to induce immune protection against schistosomes (Jiang et al., 2004). When *M. fortis* splenic cells were incubated with schistosomula for 16–18 h at 37 °C, the mortality of the schistosomula was equivalent between wild and laboratory mice (66.02% and 66.38%, respectively). Therefore, specific immune functions in *M. fortis* cells appear to play an important role in protection against schistosome infection.

BMT is an important method for treating hematological malignancies, aplastic anemia and immune dysfunction. A successful transplant can restore hematopoietic function, but immune reconstitution is not immediate. Mature T cells can be detected from the PB within 3 months of transplantation into immunodeficient patients, and T cells can reach normal levels within 6–9 months. The recovery of B cells is slower than that of T cells, as the former needs 12–18 months to reach normal levels in the PB (Lev et al., 2012). As a major animal model for BMT, allogeneic transplantation studies in mice have shown that splenic mononuclear cells can recover to normal levels within 14 days of transplantation, $CD8^+$ T cells can recover within 7 days, and $CD3^+$ and $CD4^+$ T cells can recover within 60 days. B-220^+ cells are the slowest to recover and require 60 days to reach pre-transplant levels (Liu et al., 2010).

The success of donor hematopoietic engraftment is critically dependent on the total body irradiation (TBI) dose, and allogeneic chimerism is particularly sensitive to changes in the TBI dose (Down et al., 1991). In order to establish a full donor chimeric state, a lethal dose of irradiation must be given to recipient mice. Here, nude and RAG-1$^{-/-}$ mice received a single 350–450 cGy dose from a ^{137}Cs source. In bone marrow radiosensitivity studies, animals given doses of 400 and 450 cGy died within 30 days without BMT (data not shown). Thus, the dose of 400 cGy was chosen in our current study.

All results showed that transplanted models were established successfully. However, immune reconstitution after BMT using C57BL/6 mouse-derived cells in nude and RAG-1$^{-/-}$ mice was faster than that using *M. fortis*-derived cells, which may have two possible explanations. First, *M. fortis* proteins may not be well-recognized by mouse monoclonal antibodies against IgG and CD molecules. In our preliminary research, neither rat nor mouse antibodies could bind to *M. fortis* cells (Hu et al., 2012). Second, the C57BL/6 MHC molecule is more similar to the MHC of recipient mice (nude and RAG-1$^{-/-}$) than that of *M. fortis*. It is possible, therefore, that immune reconstitution in mice receiving a BMT

Table 2
Parasite indexes detection in transplanted models.

Experiment	Groups	The number of total worms	The number of eggs per gram of liver (EPG)	The number of eggs per female worm (EPF)
Experiment 1	A. Nude mice (transplanted from *M. fortis*)	18.33 ± 2.52	42061.38 ± 3152.99[a,b,c]	10511.72 ± 3088.02
	B.Nude mice (transplanted from C57BL/6 mice)	21.00 ± 2.65	79821.75 ± 22086.09	21106.51 ± 9714.49
	C. Non-transplanted nude mice	22.67 ± 5.51	63181.49 ± 3631.32	17350.24 ± 7158.07
	D. Normal C57BL/6 mice	20.00 ± 1.73	63905.49 ± 1459.98	12545.27 ± 1712.65
	E. Normal *M. fortis*	/	/	/
Experiment 2	A. RAG-1$^{-/-}$ mice (transplanted from *M. fortis*)	18.75 ± 6.65	55455.07 ± 39363.09	11144.45 ± 2872.91
	B. RAG-1$^{-/-}$ mice (transplanted from C57BL/6 mouse)	13.50 ± 10.61	84445.48 ± 20046.77[b]	29315.87 ± 15517.35[b]
	C. Non-transplanted RAG-1$^{-/-}$ mice	21.00 ± 5.59	61842.94 ± 31375.07	10434.55 ± 4778.1

Note: a, $p < 0.05$, compared to group B; b, $p < 0.05$, compared to group C; c, $p < 0.05$, compared to group D.

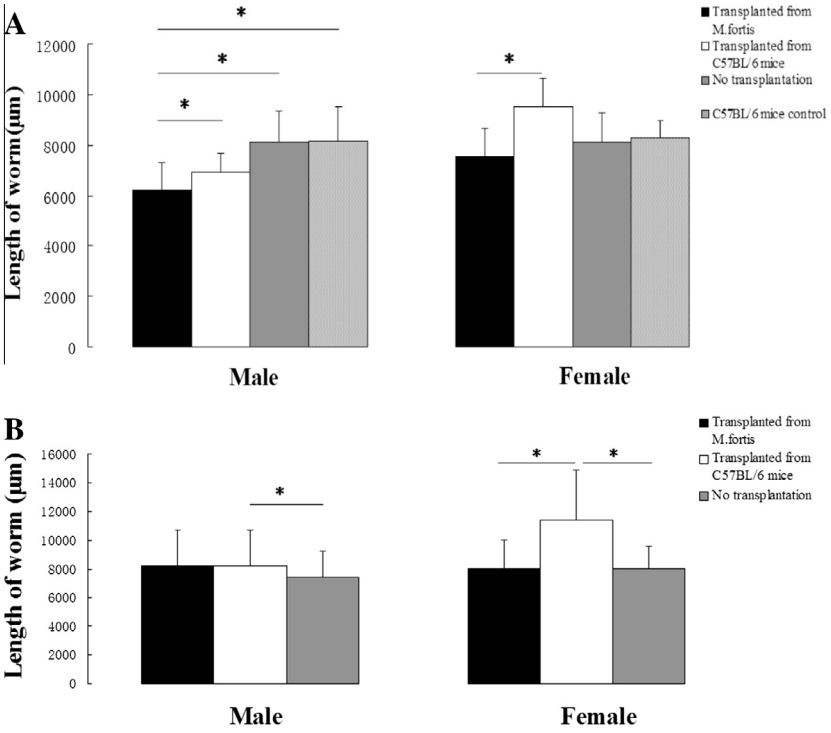

Fig. 3. Worm lengths at 6 weeks post-infection in BMT recipient mice. Adult worms were recovered by perfusion of the mesenteric and hepatic portal veins of recipient mice at 6 weeks post-infection. Worm lengths were measured and compared by Axioplan 2 imaging. (A) In the nude mice, female and male worms collected from group A were shorter than those from other groups. (B) In RAG-1$^{-/-}$ mice, female and male worms collected from group B were longer than those from other groups. On average, worms in group A were shorter than those in group B. $^{*}P < 0.05$.

from C57BL/6 mice occurred earlier than that in the group receiving *M. fortis*-derived bone marrow. Linderman and colleagues found that eliminating immune-suppressing graft-versus-host reactions permitted a superior level of immune reconstitution (Linderman and Shizuru, 2011). In their study, recipients of MHC-matched bone marrow produced T-dependent antibody responses equivalent to those of untransplanted controls by 6 weeks post-transplantation, while recipients of MHC-disparate bone marrow did not generate T-dependent antibody responses until 12 weeks post-transplantation.

In the group receiving an *M. fortis* transplant in this study, the splenic index was higher, the worm genitalia was more mature, the granuloma area was larger and the density of lymphocytes was higher compared to control RAG-1$^{-/-}$ mice. These results showed that the BMT from *M. fortis* was successful. The comparison of worms suggests that the immune function of *M. fortis* could suppress the worm size in the transplanted groups. Nude mice that received a BMT from *M. fortis* contained shorter worms than those in both control mice and nude mice that received a BMT from C57BL/6 mice. Likewise, RAG-1$^{-/-}$ mice that received a BMT from *M. fortis* also contained shorter worms than mice that received a BMT from C57BL/6 mice, although worms in both BMT groups were longer than those in control mice. These results suggest that lymphocytes from *M. fortis* can affect the growth and development of the parasite. Previous observations have suggested that IL-7 and thyroxine play synergistic roles in parasite establishment and development. Infection of mice deficient in IL-7 led to parasite dwarfism. Interestingly, mice treated with thyroxine had increased worm numbers and developed very large worms, whereas parasites infecting mice on an iodine-deficient diet displayed reduced maturation, egg laying and liver pathology (Saule et al., 2002). Further study is needed to determine if the decreased worm sizes after the *M. fortis* BMT were related to IL-7 or thyroxine levels.

The microscopy analysis suggests that the immune function of *M. fortis* can suppress the growth and development of the worms. In the nude mice, the EPG and EPF in mice that received a BMT from *M. fortis* were lower than those in other groups. By morphology, we showed that in the group receiving the *M. fortis* BMT, densities of ovarian and testicular reproductive cells of the worms were low, and outlines of ovaries and testicles were unclear. Meanwhile, the reproductive development of worms in the other groups was more advanced. In RAG-1$^{-/-}$ mice, the reproductive system of worms in the *M. fortis* BMT group was improved over the control but was inferior to that in the C57BL/6 BMT group. These results suggest that BMT is beneficial for worm development, but *M. fortis* lymphocytes may also damage the worms. Previous reports have shown that viable egg production by parasites in IL-7R$^{-/-}$ mice is severely compromised, and TGF-β signaling plays a major role in this process (Freitas et al., 2007). Another study showed that protein tyrosine kinases (PTKs) play key roles in the regulation of gonad development, eggshell gene expression and, consequently, egg production (Knobloch et al., 2006). Whether the immunological components of *M. fortis* can suppress the expression of TGF-β and PTKs to restrict the development of schistosomes needs to be studied further.

The RAG-1 recombinase enzyme can catalyze V(D)J recombination of immunoglobulin and T-cell receptor genes. RAG-1-deficient mice have small lymphoid organs that lack mature B and T lymphocytes, and the RAG-1$^{-/-}$ immune system can be described as that of non-leaky SCID mice (Mombaerts et al., 1992). Schistosome eggs exit the human host facilitated by functional responses, and the efficacy of this process decreases in schistosomiasis patients who are co-infected with HIV (Karanja et al., 1997). Upon passive transfer of CD4^{+} T cells into RAG-1$^{-/-}$ mice early during infection, the survival of the parasite was shown to be prolonged (Davies et al., 2001). Most strains of nude mice are slightly "leaky" and do have a few T cells, especially as they age. For this reason,

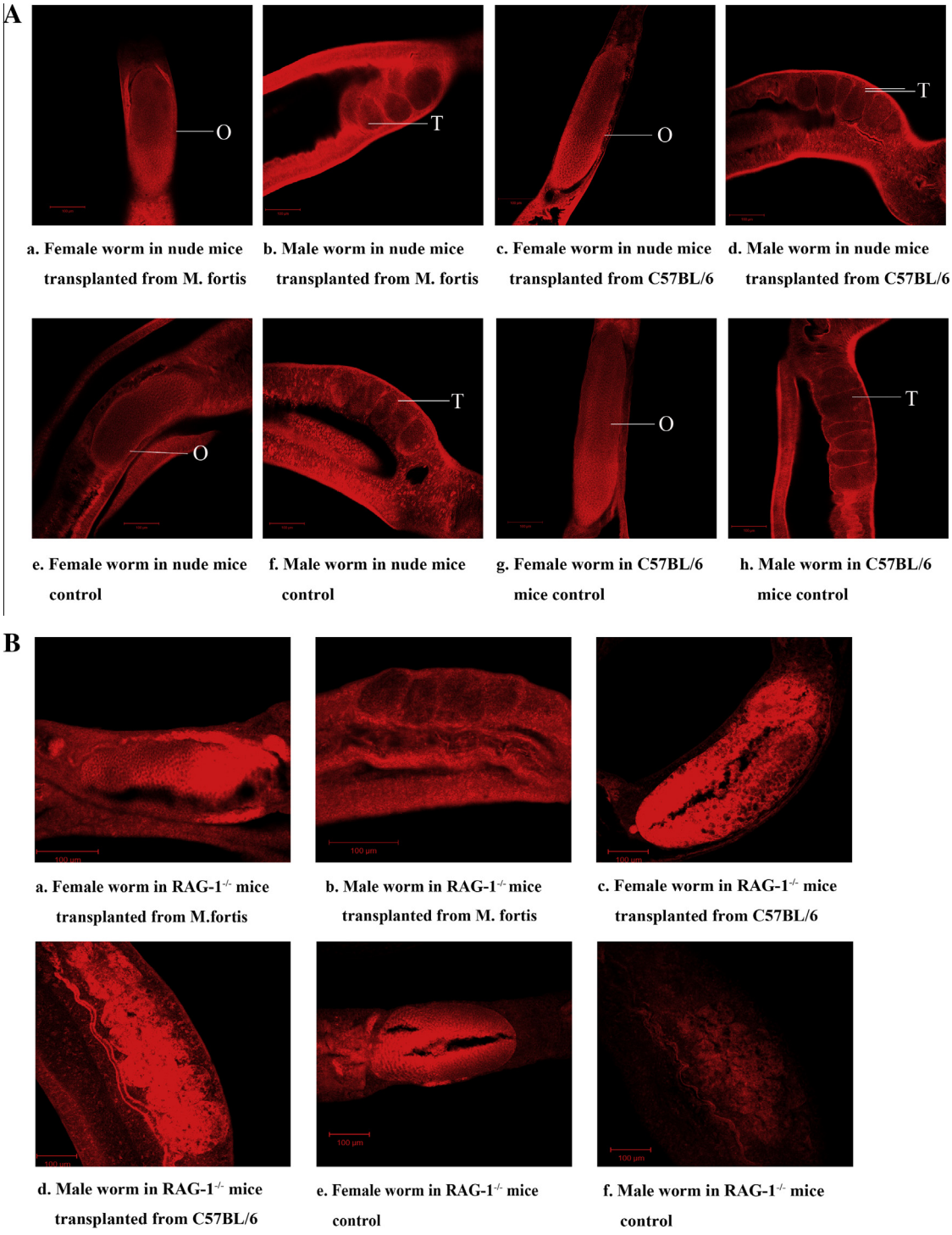

Fig. 4. Development of reproductive systems in worms collected from BMT recipient mice. Ovaries (O) and testicular lobules (T) from worms in various groups were compared by laser scanning confocal microscope. (A) In nude mice, significantly fewer oocytes and spermatocytes were in worms from group A than in the other groups, and contours of the ovaries and testes were unclear. (B) In RAG-$1^{-/-}$ mice, outlines of worm testes and ovaries were more clear in group B than those of groups A and C. In general, the reproductive systems of worms in group B were more well-developed than those in group A.

RAG-$1^{-/-}$ mice are used for their enhanced defects in the immune system. This difference is likely the reason for the improved schistosome growth and development in the nude mice controls compared with the RAG-$1^{-/-}$ mouse controls.

No differences in liver granuloma sizes were observed in three groups of nude mice. Granulomas in RAG-$1^{-/-}$ mice that received a BMT from *M. fortis* were significantly larger than those of mice receiving a C57BL/6 BMT or the control. However, mice receiving a C57BL/6 BMT also had larger granulomas than the control group. This result suggests that the size of the granulomas correlated both with the lymphocytic density and immunological characteristics of the host. Previous studies have shown that, early during schistosome infection, IL-4 increased significantly more in *M. fortis* than in mice (Zhang et al., 2000). Furthermore, high levels of IL-4 were found to be crucial for recruiting eosinophils (Chensue et al., 1994) and inducing large granulomas. These factors may explain why

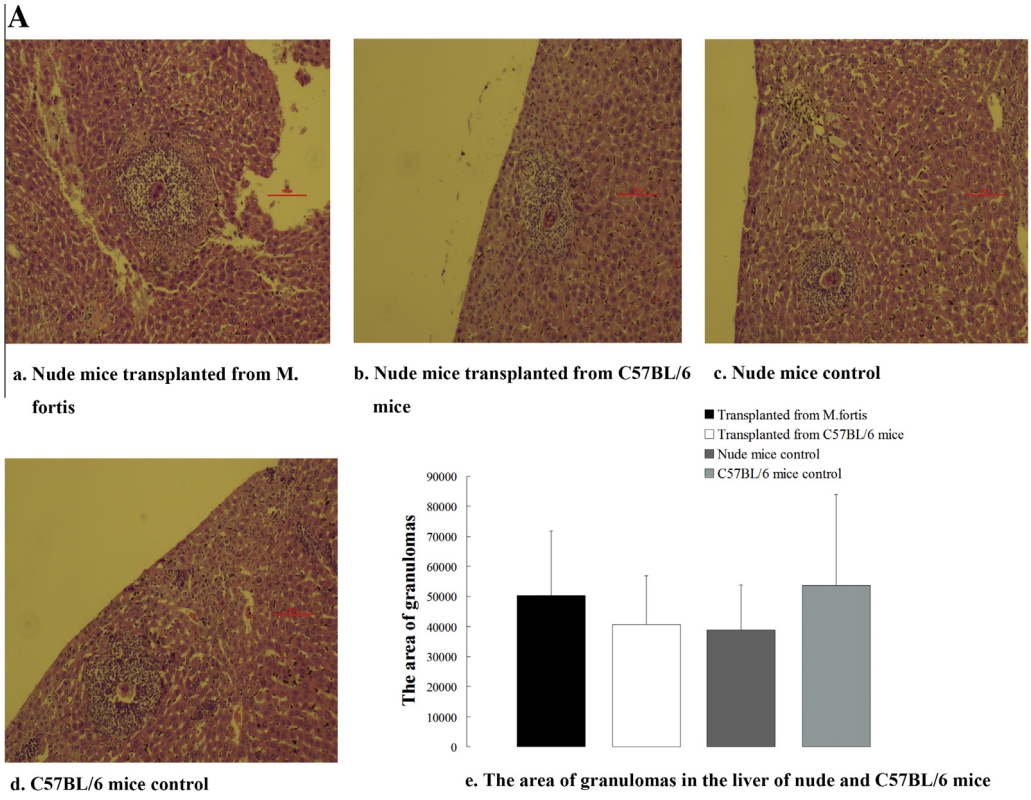

Fig. 5. Characterization of granulomas in the liver of BMT recipient mice. The area surrounding granulomas was measured using Image Pro Plus, version 3.0, to evaluate pathological changes in transplanted mice at 42 days post-infection. (A) No significant difference was observed between the transplanted and non-transplanted nude mice. (B) In RAG-1$^{-/-}$ mice, the area of granulomas in group B was larger than that in group C. The area of granulomas in group A was significantly larger than that in group B.

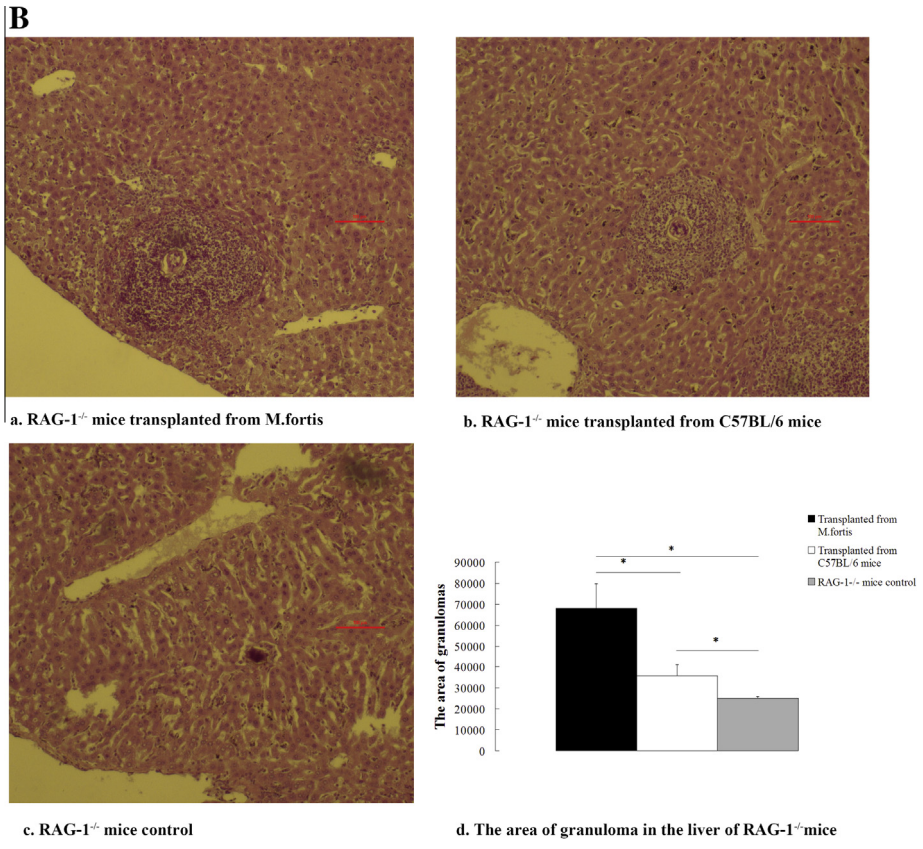

Fig. 5 (*continued*)

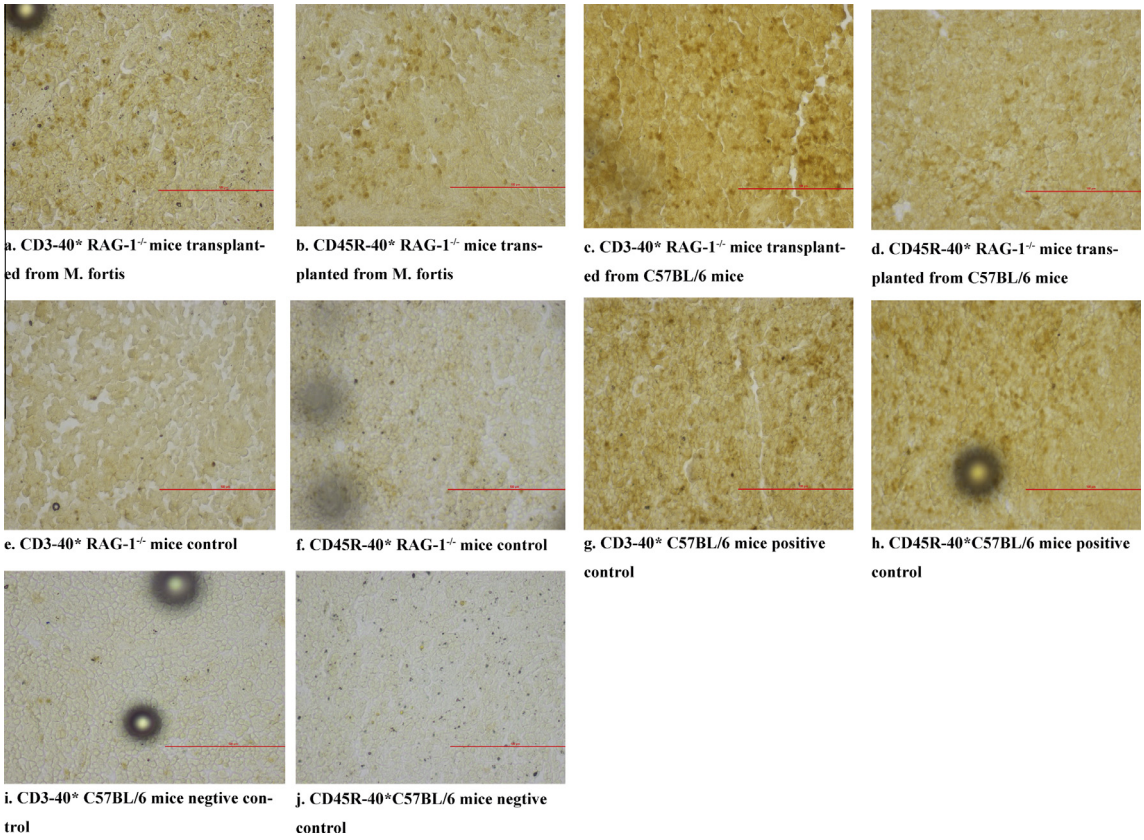

Fig. 6. Density of splenic lymphocytes in RAG-$1^{-/-}$ and C57BL/6 mice. The density of splenic lymphocytes in RAG-$1^{-/-}$ and C57BL/6 mice was detected by immunohistochemistry. In RAG-$1^{-/-}$ mice, numbers of T and B lymphocytes were significantly increased in the transplanted groups. The proportion of lymphocytes in group B was higher than that in group A.

mice receiving the BMT from *M. fortis* had the largest granulomas in this study.

5. Conclusion

In our study, immunological functions of immunodeficient nude and RAG-$1^{-/-}$ mice were successfully reestablished by BMT with cells from *M. fortis* and C57BL/6 mice. The growth and development of worms collected from mice with *M. fortis*-derived transplants were deficient compared to those with mouse-derived transplants. Our results suggest that *M. fortis* immune factors can negatively impact the size and reproductive system of schistosomes, thus playing an important role in the development of the parasite.

Authors' contributions

Conceived and designed the experiments: Y.H. and J.C.; Performed the experiments: Y.H., Y.X., W.L., H.Q., Y.S., Z.Y., W.Z., J.Z. and Y.H.; Analyzed the data: Y.H. and J.C.; Contributed reagents/materials/analysis tools: Y.H. and J.C.; Wrote the paper: Y.H. and J.C.

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