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ORIGINAL PAPER

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Cell-mediated response at the muscle phase of *Trichinella pseudospiralis* and *Trichinella spiralis* infections

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Abstract The cell-mediated response in BALB/c mice infected either by *Trichinella pseudospiralis* or *Trichinella spiralis* was compared at days 30–50 post-infection (muscle phase). The former species is non-encapsulated, whereas the latter is encapsulated in host muscles. The pattern of response against the two species was similar. Both species elicited T_H0 or T_H1/T_H2 response, with the last one being dominant. Productions of interferon gamma (IFN-γ), interleukin (IL)-4 and IL-5 were observed after antigenic restimulation of splenocytes from infected mice. No significant difference was observed between the levels of response to concanavalin A (Con-A) by the splenocytes from both infected and non-infected animals. There was a significant increase in serum IgG_1 and IgG_{2a}. Flow cytometric analysis revealed a marked proliferative response of splenocytes from infected mice to worm antigens, dominated by B (CD19) lymphoblasts. Only a few helper (CD4+) and cytotoxic (CD8+) T lymphoblasts were present. This was confirmed by an up-regulation of CD69, with a dominant expression on B lymphoblasts. In conclusion, the minimal or lack of intense cellular response against *T. pseudospiralis* in muscles is likely not due to depression of cell-mediated immunity.

Introduction

Most immunological studies on trichinellosis have mainly been focused on the intestinal phase of *Trichinella spiralis* (Wakelin and Grencis 1992; Wakelin et al. 1994; Ishikawa et al. 1998; Urban et al. 2000; Vallance et al. 2000). This is probably related to the general interest on mucosal immunity. However, little is known about the role of cell-mediated response at the muscle phase of worm development. Majority of reports in the literature described the general cellular infiltrations, myopathology and nurse cell formation (Gabryel et al. 1995; Stewart 1995; Wu et al. 2005).

Unlike *T. spiralis*, the infective-stage larva of *Trichinella pseudospiralis* is not enclosed by a collagenous capsule in muscles, yet eliciting little cellular response from the host (Kramer et al. 1981; Stewart 1995; Li and Ko 2001). Such adaptive mechanism(s) is unknown. One suggestion is that the worm can induce secretion of corticosterone by the host, leading to immunodepression (Stewart et al. 1988; Stewart and Larsen 1989; Larsen et al. 1991; Boles et al. 2000). Bolaz-Fernandez and Wakelin (1990) and Shupe and Stewart (1991) suggested that antigens of the infective-stage larvae could suppress the chemotactic response of neutrophils. In our previous study, Li and Ko (2001) compared the inflammatory response during the muscle phase of the two *Trichinella* species by a combination of mixed oral infections or injections of newborn larvae into mice of different immunocompetencies. In mixed infections, there was no reduction in the intense cellular infiltrations around *T. spiralis*. This observation could imply that the "anti-inflammatory" adaptation of *T. pseudospiralis* involves a specific and localized mechanism which cannot affect *T. spiralis*.

The present study represents our further efforts to elucidate the mechanism of host–parasite interactions of *T. pseudospiralis* at the muscle stage. The following parameters in *T. pseudospiralis* and *T. spiralis* infections were compared: expression profiles of cytokines, switching of antibody isotypes, lymphocytic proliferative responses to worm antigens and immunophenotypes.

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Materials and methods

Parasite and experimental infection

The strain of *T. spiralis* used was originally isolated by R. C. Ko from an infected pig in Guelph, Ontario, Canada, in

1967 and has since been maintained routinely in the laboratory by serial passages through Wistar rats and ICR mice. The strain of *T. pseudospiralis* was kindly given by Prof. Donald Lee, formerly of the School of Biology, The University of Leeds, UK.

Female BALB/c mice, 8–12 weeks old, were used for experiments. They were obtained from the Laboratory Animal Unit of the University of Hong Kong. Three groups (7–10 animals/group) of animals were used for either *T. pseudospiralis* or *T. spiralis* infection. Each mouse was fed orally with about 500 infective-stage larvae by Pasteur pipette. The larvae were recovered from muscles of experimentally infected ICR mice by the standard pepsin digestion method using a Baermann's funnel. The infected BALB/c mice were killed by giving an overdosage of anaesthetic ether at days 30, 40 and 50 post-infection. These periods were chosen to ensure that all the larvae in muscles would be fully developed at the time of the experiment. The same number of uninfected mice served as the negative control.

Somatic antigens preparation and collection of antiserum

After recovery, the infective-stage larvae were washed five times in saline before being homogenized on ice by a homogenizer (Ultra-TurraxT25). The homogenate was allowed to extract overnight at 4°C. It was centrifuged (Eppendorf) for 30 min at 960×*g* and 4°C.The supernatant was centrifuged for 30 min at the same temperature. Protein concentrations were determined by the BioRad protein assay kit. The results were read by a spectrophotometer (Shimadzu UV) at 595 nm. Blood samples collected from infected mice (using a 23-gauge needle and syringe) were allowed to clot overnight at 4°C and centrifuged for 10 min at 1,000×*g*. Both crude somatic antigens and sera samples were stored at −20°C until use.

Harvesting of splenocytes

Spleens from infected and uninfected mice were placed on a nylon mesh in a Petri dish containing the following: 10 ml of RPMI-1640 medium (Gibco), supplemented with 0.25 μmg/ml of amphotericin B (Sigma), 100 U of penicillin, 100 μg/ml of streptomycin (Gibco), 2 mM L-glutamine, 5×10^{-5} 2-mercaptoethanol (Sigma) and 5% heat-inactivated foetal bovine serum. The spleens were teased apart by a pair of fine forceps. The plunger of a 5-ml syringe was used to press pieces of spleen against surface of the nylon mesh (in a circular motion) until only fibrous tissues remained. The cell suspension was centrifuged at 240×*g* for 5 min at room temperature (RT). After discarding the supernatant, the cell pellets were re-suspended in 40 ml of ACK lysing buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA). The cell pellets were washed twice in RPMI-1640 medium. After centrifugation, the cells were suspended at a density of 2×10^7 cells/ml in the same medium. The number of splenocytes was counted by a haemocytometer.

Lymphocyte proliferation assay

Splenocytes were seeded in triplicates in 96-well polystyrene culture plates at 5×10^5 cells/well. Cells were stimulated with 10–50 μg/ml of worm antigens for 96 h (antigenic restimulation) and with 2–4 μg/ml of concanavalin A (Con-A; Sigma) for 48 h in an incubator at 37°C and 5% carbon dioxide. Non-stimulated cells served as background control.

For antigenic restimulation, after 76-h incubation, the cultures were pulsed with 1 U Ci of [^{3}H]thymidine (Amersham). For Con-A stimulation, the cells were pulsed with an equal amount of [H^3] thymidine for additional 18 h. The splenocytes were harvested onto glass micro-fibre filters (Whatman) using a lymphocyte cell harvester (Dynatech). The uptake of tritiated thymidine was measured in liquid scintillation cocktail using a liquid scintillation counter (Beckman).

Cytokine assay

After in vitro antigenic restimulation for 96 h, supernatants collected from 96-well culture plates were measured for tumour necrosis factor alpha (TNF-α), interleukin (IL)-4 and IL-5. For interferon gamma (IFN-γ) measurements, supernatants were collected at 48 h after restimulation. For the Con-A experiment, supernatants collected at 48 h were measured for cytokines.

Concentrations of the above cytokines were estimated using a sandwich ELISA kit (Pharmingen). The protocols mainly followed those provided by the manufacturer, with slight modifications. Polystyrene micro-titration plates (Maxisorp, Nunc) were coated with monoclonal antibodies against the cytokines at the following dilutions: IFN-γ, 1:2,000; IL-4 and IL-5, 1:250; TNF-α, 250. The non-specific binding sites were blocked by 1% bovine serum albumin (BSA) for 1 h. Biotinylated monoclonal antibodies (1:250) were used as the secondary antibodies. Avidin–horseradish peroxidase (HRPO) was used as the enzyme and tetramethylbenzidine (TMB) served as the substrate. For IFN-γ determination, plates were incubated in the dark for 10 min at RT for colour development. For TNF-α, IL-4 and IL-5 determinations, the incubation time was extended to 30 min. The level of cytokine was quantified using the standard curve of a known amount of recombinant cytokine. The sensitivities of these assays (picograms per millilitre) were 25 for IFN-γ, 15.6 for TNF-α, 7.8 for IL-4 and 15.6 for IL-5.

Measurement of serum antibodies

Indirect ELISA was used to monitor the levels of IgG_1 and IgG_{2a} in serum samples. The optimal dilutions of various

reagents were first determined by checkerboard titrations. Each well of a polystyrene micro-titration plate was coated with 100 μl (1 μg/ml) of crude somatic antigens of worms. After washing, the non-specific binding sites were blocked by 1% BSA. The wells were washed three more times before addition of 100 μl of the test serum (diluted 1:100). The plate was incubated for 2 h at RT and then washed. One hundred microlitres of 1:1,000 HRPO-conjugated rat anti-mouse antibody IgG_1 or IgG_{2a} (Pharmingen) was added into each well. The plates were incubated overnight at 4°C. After washing and incubating with TMB for 30 min at RT, the absorbance of the samples was read by an ELISA reader (Anthos) at 450 nm (test filter) and 620 nm (reference filter).

Flow cytometric analysis

The following markers from Pharmingen were used: CD4-FITC (clone GK.1.5), CD-PE/FITC (clone 53-6.7), CD19-FITC (clone ID 3), CD25-PE (clone PC61) and CD69-PE (clone H.2F3). For staining activation markers (CD25-PE and CD69-PE), PE-conjugated rat IgG_1 (clone A110-1) and PE-conjugated hamster IgG group 1λ (clone G235-2356) served as isotypic control. This was undertaken to define positions of quadrant markers (dot plots) of positive staining.

Splenocytes isolated from infected or uninfected mice were seeded in flat-bottom 96-well polystyrene culture plates at 5×10^5 cells/well, in triplicates. The cells were stimulated with 25 μg/ml of somatic worm antigens for 48–96 h in a carbon dioxide incubator at 37°C. The splenocytes were transferred into 12×75 mm polypropylene tubes (Elkay) and centrifuged for 5 min at 240×*g* and RT. The cell suspensions were washed twice using a buffer (1% BSA and 0.1% sodium azide) before being stained for surface markers. For staining activation markers on B (CD19) and subsets of T (CD4/CD8) lymphocytes, 1.5×10^6 splenocytes were re-suspended in 50 μg of staining buffer and placed on ice with anti-mouse CD16/CD32 for 5–10 min. This was followed by addition of 0.25 μg of mAbs (CD4/CD8/CD25/CD69). The sample was maintained on ice in the dark for 30 min. For staining B lymphocytes, 0.1 μg of CD19 mAb was used.

After surface staining, the splenocytes were washed two times in 1 ml of staining buffer. The re-suspended cells were analysed by a flow cytometer (Epics Elite, Beckman Coulter). For optical alignment, Flow-Check fluorospheres (Beckman Coulter) were used. Daily variations in fluorescent intensities of FL1 (FITC) and F2 (PE) were standardized by using Immuno-Brite standard kits (Beckman Coulter). A total of 10,000 events were counted and analysed. Dead cells and cell debris were excluded, based on the dot plots of forward scatter (FSC) against side scatter (SSC) in linear amplification. List mode data were analysed by WinMD 12.8 (Scripps Institute). The expression level of CD25 was interpreted as median fluorescent intensities (MFI). The expression level of CD69 was interpreted as the percentage of lymphocyte subsets showing up-regulation of activation marker.

Statistical analysis

Unpaired two-tailed Student's *t* test was used to compare the cytokine data. One-way analysis of variance (ANOVA)

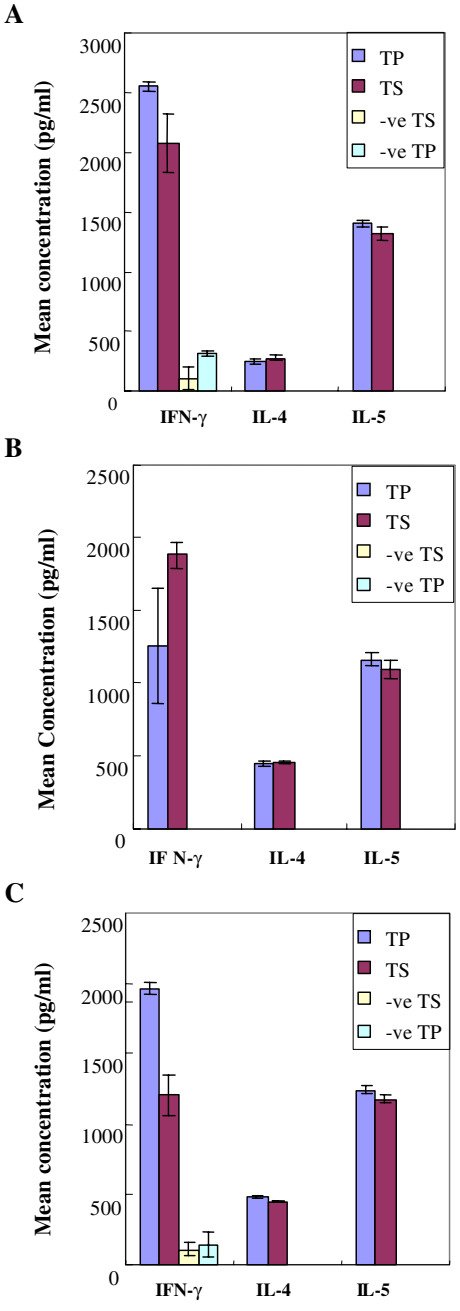


Fig. 1 **a** Production of cytokines from splenocytes of BALB/c mice infected with *Trichinella pseudospiralis* (*TP*) or *Trichinella spiralis* (*TS*) after stimulated with 25 μg of somatic antigens at day 30 post-infection. **b** At day 40 post-infection. **c** At day 50 post-infection. The data were based on results of three experiments. *-ve TS* uninfected control for *T. spiralis*, *-ve TP* uninfected control for *T. pseudospiralis*

was used to evaluate the lymphocyte proliferation data. A p value of less than 0.05 was considered statistically significant.

Results

Cytokines

As compared to the negative controls, significant levels of IFN-γ, IL-4 and IL-5 were detected in the supernatant of splenocytes from mice infected with either *T. pseudospiralis* or *T. spiralis* after in vitro antigenic restimulation (Fig. 1). This indicates a T_H0 or T_H1/T_H2 response. For *T. pseudospiralis* infection, the mean concentration of IFN-γ on day 40 post-infection was lower than that on days 30 and 50. For *T. spiralis* infection, the mean concentration of IFN-γ on day 50 was lower than that on days 30 and 40. Similar fluctuations were not observed for IL-4 and IL-5. However, the levels of the former were lower than those of the latter.

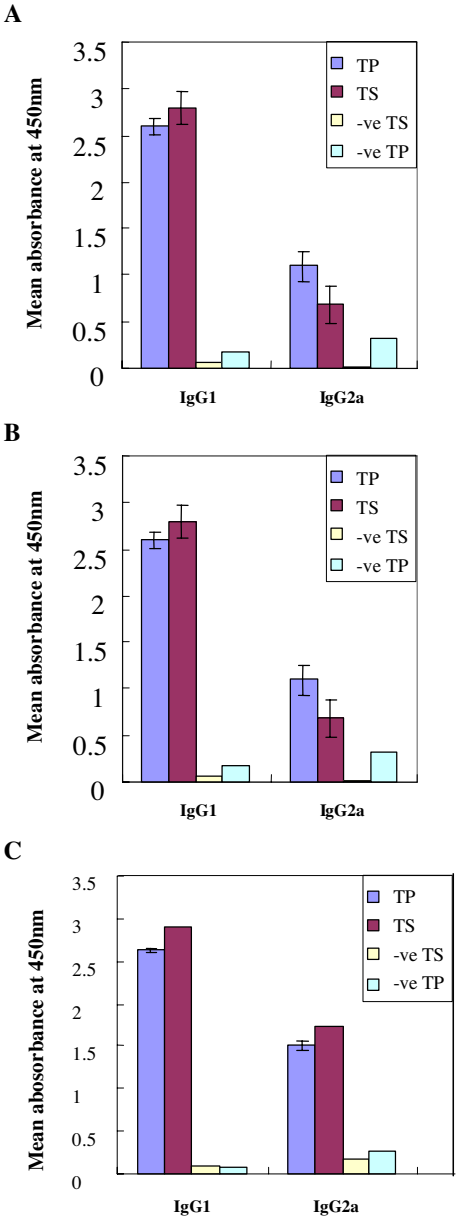


Fig. 2 **a** Presence of IgG_1 and IgG_{2a} in serum of BALB/c mice infected with *T. pseudospiralis* (*TP*) or *T. spiralis* (*TS*) at day 30 post-infection. **b** At day 40 post-infection. **c** At day 50 post-infection. *Statistically significant from negative control at $p<0.01$. *-ve TS* Uninfected control for *T. spiralis*, *-ve TP* uninfected control for *T. pseudospiralis*

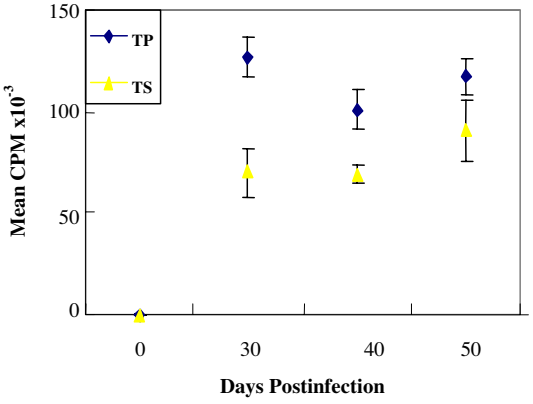


Fig. 3 Results of in vitro lymphocyte proliferative response from BALB/c mice infected with *T. pseudospiralis* (*TP*) or *T. spiralis* (*TS*) at days 30, 40 and 50 post-infection. The splenocytes were stimulated by 25 μg of worm antigens. *Statistically significant from control at $p<0.01$, **statistically significant from control at $p<0.05$. The reading at day 0 represents that of negative control

Antibody isotypes

Significant levels of IgG_1 and IgG_{2a} were observed in the serum of mice infected either with *T. pseudospiralis* or *T. spiralis*. For both infections, the level of IgG_{2a} was higher on day 40 than that on days 30 and 50 (Fig. 2). The high concentrations of the two immunoglobulins in infected serum samples indicate a predominance of the "type 2" immunity.

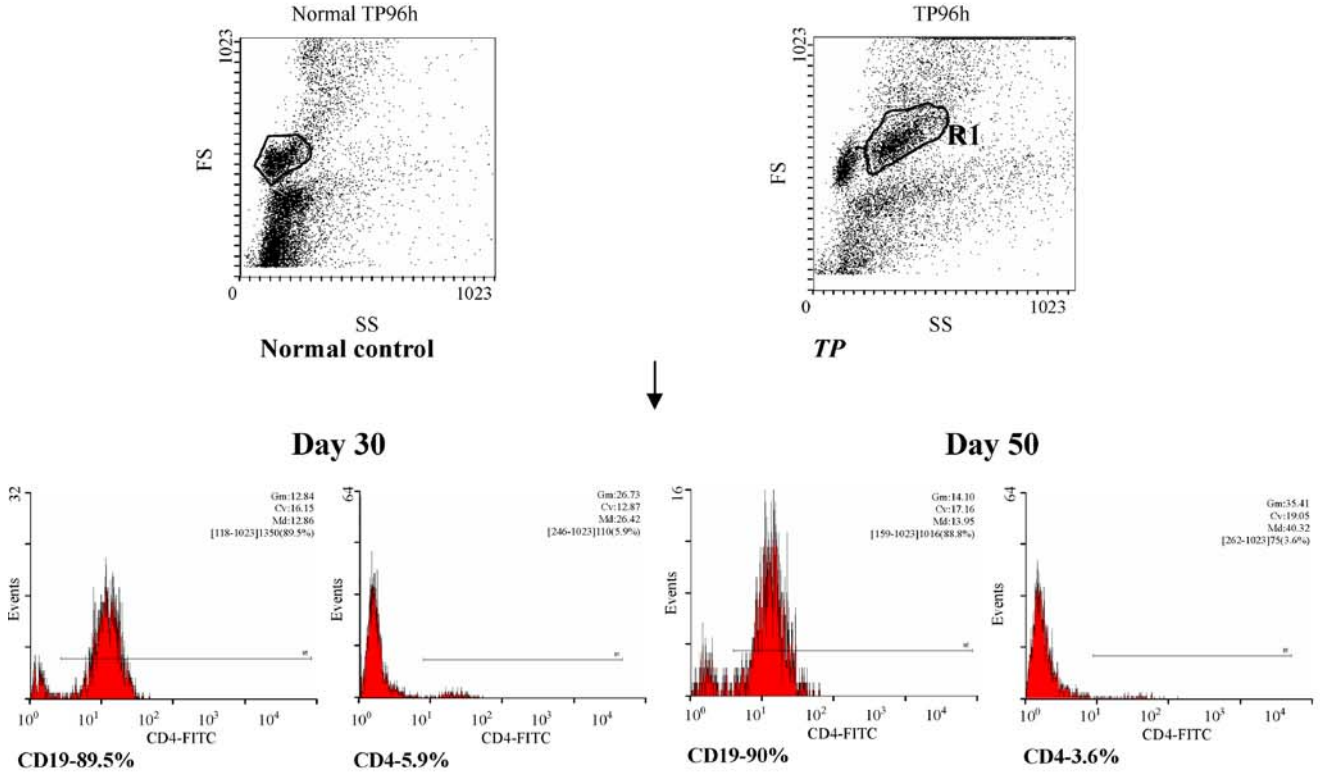
Lymphocyte proliferative response

Based on radioactive labelling and liquid scintillation counting, the lymphocyte proliferative response of cells from infected animals was significantly higher than that of the negative control. However, the level of response in cells from *T. pseudospiralis* infection was higher than that of *T. spiralis* ($p<0.05$) at all the three experimental periods post-infection, i.e. days 30, 40 and 50 (Fig. 3).

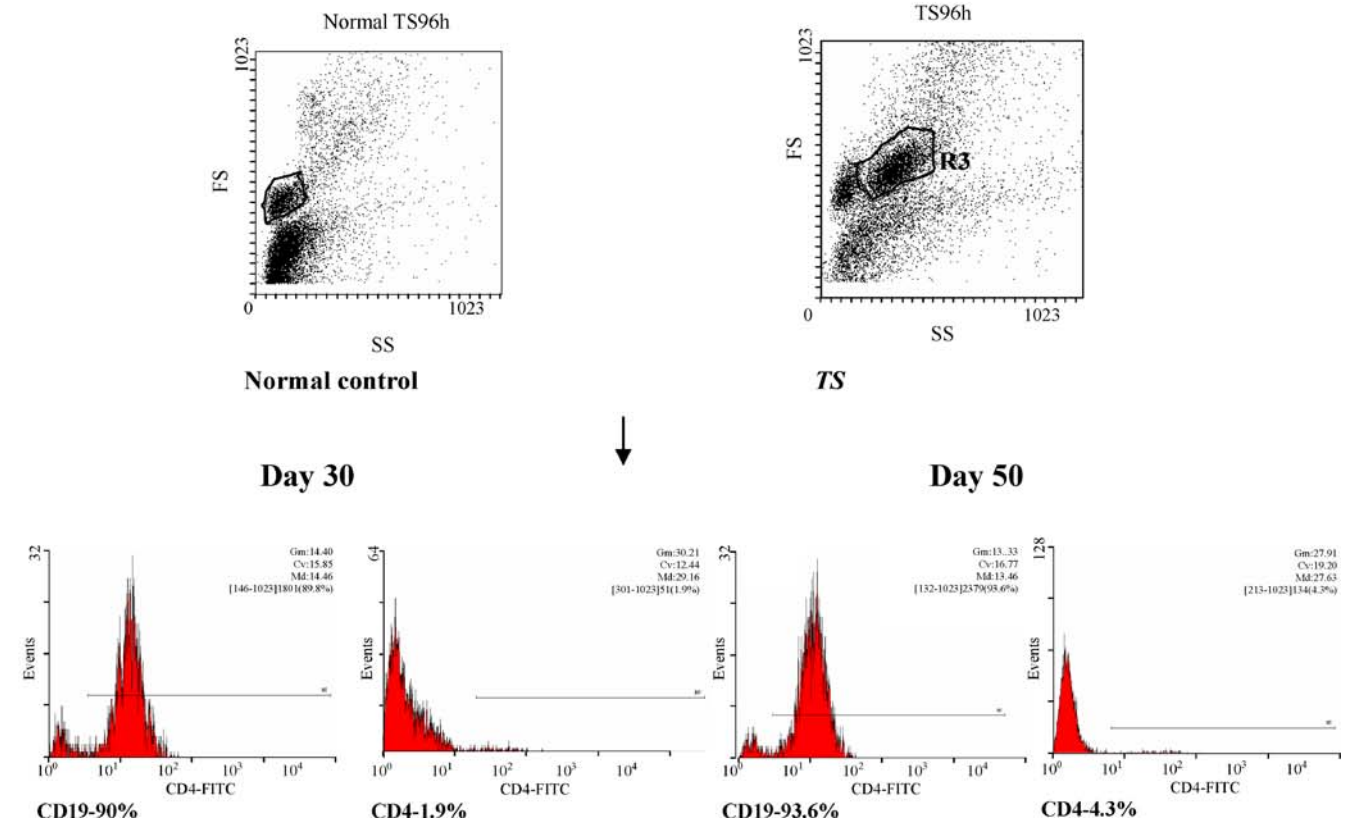
Flow cytometric analysis

At 48 h after antigenic restimulation of splenocytes (from infected mice) with worm antigens, signs of blastogenesis were observed in the gated region (R1 and R2) of

T. pseudospiralis (Day 30 and 50)



T. spiralis (Day 30 and 50)



◀ **Fig. 4** Results of flow cytometric analysis of lymphocytes after restimulation with *T. pseudospiralis* (*TP*) or *T. spiralis* (*TS*) antigens on days 30 and 50 post-infection. A distinct region of lymphoblasts (TP=R1, TS=R3) was gated for analysis of expression of cell-surface markers (CD4, CD8, CD19, CD25, CD69). The data show the dominance of B lymphoblasts (CD19) and presence of few T cells (CD4, CD8). Note the absence of R1 and R3 in the cells of negative control

lymphoblasts. This region of lymphoblasts was characterized by greater signals of SSC. At 96 h of restimulation, a distinct population of lymphoblasts (R1 and R3) was identified in the FSC vs SSC dot plots (Fig. 4). This was not observed in the negative control samples.

A majority region of lymphoblasts in the FSC vs SSC dot plot was consisted of B lymphoblasts. Few CD4+ and CD8+ T cells were present. The number of B lymphoblasts in *T. pseudospiralis* infection was similar to that of *T. spiralis* infection, so as the number of CD4+ and CD8+ cells.

The activation of B lymphoblasts was further confirmed by the experimental result of CD69 (early activation marker) expression on the cell surface. At 48 and 96 h after antigenic restimulation, B lymphoblasts were found to be dominant (among the proliferated cell population) in expressing this marker (2.8–7.9%). This was substantially higher than the 0.25–1.32% observed for T cells (Table 1).

Response to Con-A

Splenocytes from both infected mice (*T. pseudospiralis* or *T. spiralis*) and non-infected animals showed a marked proliferative response to Con-A (at 2–4 μg/ml concentration) (Fig. 5). However, there was no significant difference between the level of response. A similar result was also observed on the production of IFN-γ (2,360–2,835 pg/ml) and IL-4 (22–306 pg/ml). The production of IL-5 (765–1,180 pg/ml), however, was only limited to splenocytes from infected animals. The expression of TNF-α was not observed.

Table 1 Results of the expression profiles of CD69 on splenocytes from mice infected with *Trichinella pseudospirals* or *Trichinella spiralis* under in vitro antigenic restimulation at days 30 and 50 post-infection

Day	Infection	Incubation time (h)	CD19+CD69+ B cells (%)	CD4+CD69+ T cells (%)
30	TP	48	7.92±2.60 (0.12)[a]	0.90±0.400 (0.04)[a]
	TS		6.00±3.40 (0.30)	0.68±0.270 (0.04)
30	TP	96	3.00±0.50 (0.27)	0.25±0.025 (0.00)
	TS		2.80±0.50 (0.06)	0.50±0.050 (0.09)
50	TP	48	2.00±0.65 (0.11)	0.56±0.160 (0.00)
	TS		2.95±0.30 (0.07)	0.56±0.130 (0.02)
50	TP	96	7.27±0.13 (0.34)	0.65±0.020 (0.00)
	TS		7.31±1.44 (0.32)	1.32±0.150 (0.19)

The data were based on the mean and SEM of three experiments
TPTrichinella pseudospiralis, *TSTrichinella spiralis*
[a]Isotypic control data

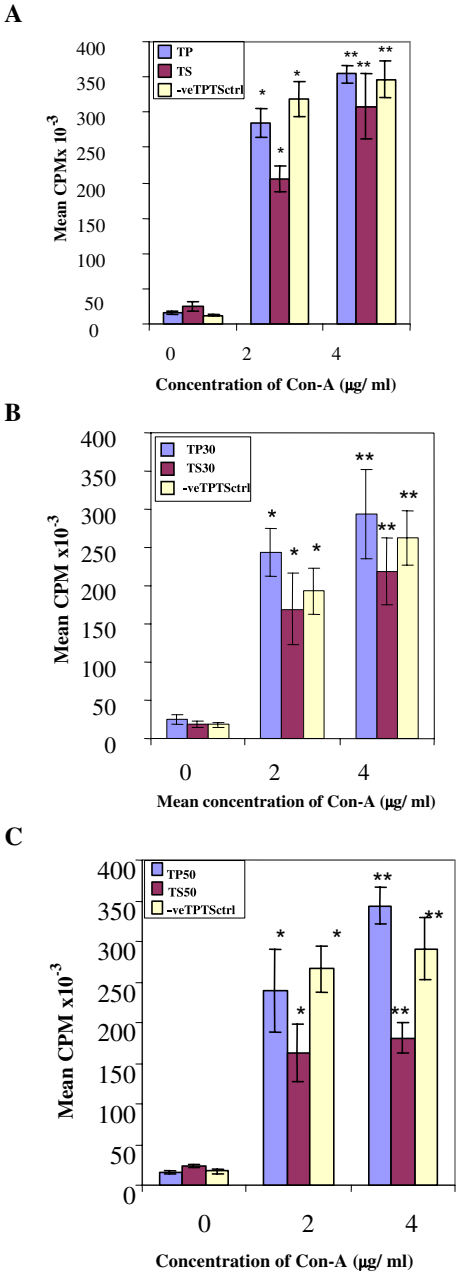


Fig. 5 **a** Result of in vitro T cell proliferative response to Con-A at day 30 post-infection with Trichinella pseudospiralis (TP) and T. spiralis (TS). **b** At day 40 post- infection. **c** At day 50 post-infection. Splenocytes from infected mice were stimulated with 2 or 4 μg/ml of Con-A for 48 h. The cultures were then pulsed with 1 μ Ci of tritiated thymidine/well of culture plate for additional 18 h. * Statistically significant from control at $p < 0.05$. ** Statistically significant from control at $p > 0.05$. –veTPTSctrl = uninfected control animals Lee and Ko- Cell-mediated response

Discussion

The present study has clearly demonstrated that both *T. pseudospiralis* and *T. spiralis* elicited a similar pattern of cell-mediated response at the muscle phase of development. There was no substantial difference in the profile of in vitro expression of cytokines, switching of antibody

isotypes, lymphocytic proliferative response and response to Con-A. Therefore, the data do not support the hypothesis that the non-encapsulated *T. pseudospiralis* evades the cellular response in muscles by a systemic depression of the host immunity.

A lower level of IFN-γ production (after stimulation of splenocytes with worm antigens) was observed in *T. spiralis* infection at day 50 than at days 30 and 40 post-infection. Similarly, a lower level of IFN-γ was observed in *T. pseudospiralis* infection at day 40 than at days 30 and 50 post-infection. However, since the standard errors of the means of some data were fairly large, we attribute these variations to differences in individual host response. The same interpretation applies to the data on the levels of IgG_1 and IgG_{2a} in serum.

In the lymphocyte proliferative response experiment, a significantly higher response was recorded in *T. pseudospiralis* than in *T. spiralis* infection. However, this difference may not have any biological implication other than indicating that the former species did not elicit an immunodepressive activity. This is supported by the fact that in flow cytometric analysis of the lymphocyte cultures of the two trichinellids, no difference was observed in the percentage of B lymphoblasts present.

The expression profile of cytokines (IFN-γ, IL-4 and IL-5) and antibody isotype switching (IgG_1, IgG_{2a}) indicate a T_H0 or T_H1/T_H2 immune response at the muscle phase of worm development, with the dominance of T_H2. T_H0 is a transitional stage of T_H1 and T_H2 response (Mosmann and Sad 1996). A similar observation was noted in acute and chronic *T. spiralis* infections in humans (Morales et al. 2002). Li and Ko (2001) also reported the dominance of T_H2 response during the muscle phase of *T. spiralis* after injection of live or dead newborn larvae into BALB/c mice and measured the production of cytokines by popliteal lymphocytes. The authors suggested that the dominance of the T_H2 response during the muscle phase of trichinosis may represent a specific selection by the parasite during evolution for sustained survival in tissues. The destructive granulomatous response is strongly T_H1-mediated. Therefore, by favouring a T_H2 response, the developing larvae may overcome rejection at an early stage. T_H2 cytokines have also been reported in suppressing CD8+ cells in graft rejection (Scully et al. 1997). However, this is only a speculation. To elucidate the functional significance of the T_H1/T_H2 dichotomy, further studies using cytokine knock-out mice should be undertaken.

According to the flow cytometric data, B lymphocytes dominated in the in vitro antigenic restimulation of splenocytes from mice infected with *T. psuedospiralis* or *T. spiralis*. The observation suggests that B lymphocytes rather than the T cells play a more important role in the regulation of cell-mediated response at the muscle stage of trichinellosis. The dominance of B lymphoblasts is further indicated by the marked expression of CD69 on the cell surface. Lauzurica et al. (2000) considered that such CD marker plays a role in antibody synthesis and differentiation of B lymphocytes. Harris et al. (2000) found that B-cell subsets can regulate the differentiation of naïve CD4+ T cells to T_H1 and T_H2 cells by producing polarizing cytokines such as IL-4 and IFN-γ. This indicates that B cells may regulate immune responses to infectious pathogens via production of cytokines.

A down-regulation of local inflammatory response mediated by B cells is also possible. Li and Ko (2001) reported that the inflammatory response to the muscle phase of *T. spiralis* infection in CBA/N mice (deficient in production of B-1 lymphocytes) was weaker than that in BALB/c mice. This may suggest that B-1 lymphocytes are the major effector cells. The findings by Hall et al. (1999) and Waters et al. (2000) seem to support such argument. These authors reported that intestinal inflammation and keratitis were absent in B knockout mice (deficient in both B-1 and B-2 lymphocytes) infected with *Cryptosporidium parvum* and *Onchocerca volvulus*.

Local inflammatory response in skeletal muscles can also be mediated by their potent capacity for antigen presentation (Yokoyama et al. 1997; Nagaraju 2001). Antigen-presenting cells, co-stimulatory molecules, major histocompatibility complex (MHC) I and II, mast cells, etc. are present in skeletal muscles. Therefore, skeletal muscles per se may act as a micro-environment for initiating responses to infectious agents. However, to determine whether *T. pseudospiralis* can down-regulate the immunological capabilities of muscles, further studies based on in vitro cultures are necessary.

Using immunofluorescent laser confocal microscopy, Li et al. (1999) showed that the excretory/secretory (E/S) antigens of *T. spiralis* were confined exclusively within the nurse cell complex, whereas those of *T. pseudospiralis* were widely distributed along infected myofibres and in adjacent muscles. To explain the absence of cellular response against the latter infection, Li and Ko (2001) suggested that the nature of antigens of the non-encapsulated species may play a role. This supports the proposal by Bolaz-Fernandez and Wakelin (1990) and Shupe and Stewart (1991) that the antigens of *T. pseudospiralis* could suppress the chemotactic response of neutrophils.

As compared to the muscle phase, a short period of T_H1 response was followed by a dominance of T_H2 immunity at the intestinal phase of trichinellosis (Wakelin et al. 1994; Ishikawa et al. 1998). The latter immunity was mainly mediated by CD4+ T lymphocytes (Grencis et al. 1991; Vallance et al. 2000; Helmby and Grencis 2002). Therefore, the difference in the type of dominant lymphocytes at the intestinal and muscle phases probably suggests that the mechanism for trichinellids to survive in the two distinct niches may be different.

To conclude, on the basis of our data, it seems likely that the lack of intensive cellular response against *T. pseudospiralis* at the muscle phase may be mediated by a localized and specific effector mechanism. It may also involve a down-regulation of the local response, mediated mainly by B lymphocytes or modulation of the immunobiology of muscles.

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