γδT cells do not play a major role in controlling infection in experimental cysticercosis

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SUMMARY

Protective immunity against larval *Taenia crassiceps* has been shown to rely on T cells; however, the roles of the specific subsets of T cells during infection are not known. To investigate a possible role for γδT cells, this study investigated larval infection in δ-chain knock-out C57BL/6 (deltaKO) and wild-type C57BL/6 mice. It was found that deltaKO mice and C57BL/6 mice were equally susceptible to infection suggesting γδT cells do not play a major role in protective immunity. Cytokine production by concanavalin A (ConA)-stimulated spleen cells from infected deltaKO mice and C57BL/6 mice were determined. All infected mice demonstrated an increased IL-10 production suggesting a Th1-inhibitory function. Cells from infected deltaKO mice and C57BL/6 mice did not show increases in IL-4 production. Heavily-infected C57BL/6 mice showed a decrease in IFN-γ production compared to deltaKO mice. These observations suggest that an increase in IL-10 production best correlates with a non-protective immune response. To make comparisons between *in vitro* cytokine production and systemic immune responses, cytokine levels in serum were determined. C57BL/6 mice and deltaKO mice showed increases in serum levels of IL-4 and IFN-γ at 52 days post-infection. The systemic immune response of these mice, therefore, is a mixed Th1/Th2-type response and γδT cells are apparently not responsible for the systemic increases in these cytokines.

Key words: cysticercosis, γδT cells, cytokines, immunoregulation.

INTRODUCTION

Adult tapeworms of *Taenia crassiceps* are found in canine hosts and the larval stages are found in rodent intermediate hosts. Laboratory mice can be infected by serial subinoculation of larvae into the peritoneal cavity which will establish new infections and the larvae will grow, bud and eventually produce several thousand larvae (Freeman, 1962; Sally, Chau & Freeman, 1976).

Although anti-larval antibody-mediated responses appear to be ineffective in controlling larval growth (Hermanek & Prokopic, 1989), protective immune responses have been shown to rely on T cells (Bojalil *et al*. 1993). The cytokine profile is of a typical Th2-type response and these cytokines would be expected to promote antibody-mediated immunity (Mosmann & Coffman 1989; Paul & Seder 1994; Mosmann & Sad, 1996). However, during the first 2–3 weeks of infection, an increase in ConA-stimulated IFN-γ production by spleen cells is detected (Terrazas *et al*. 1998). This increase would be expected to promote a Th1-type response (Mosmann & Coffman, 1989). Because there is a switch from a Th1-type response to a Th2-type response during infection, and since Th1-inducing drugs seem to enhance protective immunity (Bojalil *et al*. 1993), it is suggested that a Th1-type response may be effective in controlling parasite growth.

The various roles of effector cells in immunity to experimental cysticercosis are unknown. Earlier we reported that γδT cells are expanded in the body cavity of infected BALB/c mice (Toenjes *et al*. 1999), suggesting there may be an important role for γδT cells in immunity to larvae. Therefore, using δ-chain knock-out C57BL/6 (deltaKO) mice, the present study investigated the role of γδT cells in experimental cysticercosis and cytokine profiles of Th-regulatory cytokines were determined to evaluate the type of immune response produced by deltaKO and normal C57BL/6 mice.

MATERIALS AND METHODS

Mice and infections

Six-week-old female BALB/cJ, C57BL/6, and delta-chain knock-out C57BL/6 (deltaKO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The ORF strain of *Taenia crassiceps* was used for infections (Freeman, 1962). Parasites were obtained from the peritoneal cavity of female BALB/cJ mice that had been infected for 3 months and were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2·7 mM KCl, 8·1 mM Na_2HPO_4, 1·47 mM KH_2PO_4, pH 7·2) under sterile conditions. Ten small non-budding larvae approx.

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2 mm in diameter were injected intraperitoneally in 0·75 ml of PBS into mice using a 20-gauge needle. All mice were killed 52 days post-infection (p.i.) and 3 age-matched, uninfected control mice of each strain were killed the following day.

Spleen cell preparation and culture medium

Cells were cultured in RPMI-C (RPMI 1640 with L-glutamine (Mediatech, Herndon, VA) supplemented with 5×10^{-5} M β2-mercaptoethanol, antibiotics (5 U/ml penicillin, 5 μg/ml streptomycin, 10 μg/ml neomycin; Sigma, St Louis, MO) and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA)).

Spleens were macerated in 7 ml of RPMI-C and single-cell suspensions were obtained by drawing the suspension through a 23-gauge needle and expelling through a 26-gauge needle. Red blood cells were removed by hypotonic shock and the remaining cells were washed with 10 ml of RPMI-C and counted. Cell viability was consistently $> 95\%$ as determined by trypan blue exclusion.

Proliferation assays

In the wells of flat-bottom, polystyrene 96-well plates (Corning, Corning, NY), 5×10^5 cells/well were stimulated with designated concentrations of concanavalin A (ConA, Sigma, St Louis, MO), in triplicate, in a final volume of 250 μl/well. After 60 h of stimulation, 1 μCi of [^{3}H]thymidine (ICN Pharmaceuticals, Irvine, CA) was added to each well, and cells were harvested after 12 h. Thymidine incorporation was measured by liquid scintillation spectroscopy (LS-7500, Beckman Instruments Inc., Fullerton, CA). The means of triplicate assays were determined and stimulation indices (SIs) were calculated by dividing the mean of each stimulated culture by the corresponding mean of the unstimulated culture. The means of SIs for each experimental group at each concentration of ConA were then calculated.

Cytokine ELISAs

Spleen cells were stimulated with 5 μg/ml ConA, under conditions as described above for the proliferation assays. After 72 h of stimulation, supernatant fluids were collected and the concentrations of IFN-γ, IL-4, and IL-10 were measured by sandwich ELISA. Capture and detection antibodies were purchased from PharMingen (San Diego, CA) and measurements were made according to the manufacturer's instructions. Assays were performed in triplicate and means calculated for each mouse. The mean of each cytokine concentration for each experimental group was then determined.

Flow cytometry

Splenocytes were examined for the percentages of CD4$^+$, CD8$^+$, $\alpha\beta$TCR$^+$ and $\gamma\delta$TCR$^+$ cells. Phycoerythrin (PE)-labelled anti-CD4 (H129.19) and anti-CD8 (53-6.7) and fluorescein isothiocyanate (FITC)-labelled anti-$\alpha\beta$TCR (H57-537) and anti-$\gamma\delta$TCR (GL3) were used for fluorescent labelling. Two-colour analysis was done by staining for CD4 together with the $\alpha\beta$TCR or CD8 together with the $\gamma\delta$TCR. Non-specific binding was blocked with FcBlock and isotype controls included FITC-labelled hamster IgG (G235-2356) and PE-labelled rat IgG2a (R35-95) were used as isotype controls. All antibodies used for flow cytometry were purchased from PharMingen (San Diego, CA). Staining was done in 100 μl of staining buffer (PBS, 1%BSA, 0.1%NaN_3, pH 7·5) containing 1 μg of each of the appropriate antibodies (30 min, 4 °C) and washed twice with 1 ml of staining buffer. Stained cells were fixed in Ortho Permeafix (Ortho Diagnostics Inc., Raritan, NJ), analysed by 2-colour FACS (Coulter Epics XL) at the Wake Forest University School of Medicine and evaluated using WinList (Verity Software House, Inc., Topsham, ME).

Statistical analysis

For proliferation assays, the mean stimulation indices (SIs) were compared with a repeated measures, 2-factor ANOVA. Significant differences between the mean SIs for uninfected and infected mice for each concentration of ConA were compared with the Scheffé *post-hoc* test (Gravetter & Nalluau, 1996). Comparisons between infected and uninfected mice for the remaining assays were made using the Student's *t*-test.

RESULTS

Larval growth in C57BL/6 and deltaKO mice

To investigate the role of $\gamma\delta$T cells in experimental cysticercosis, wild-type C57BL/6 mice and deltaKO C57BL/6 mice were infected for 52 days and then numbers of larvae were determined. No significant differences in numbers of larvae were found between the C57BL/6 mice (296 ± 77 S.D.) and deltaKO mice (269 ± 85 S.D.). These data suggest that $\gamma\delta$T cells do not play a major role in protective immunity against larvae.

Parasite suppression of T cell proliferation

To re-examine the phenomenon of immunosuppression, spleen cells were stimulated with ConA and proliferation was quantified by [^{3}H]thymidine incorporation. Both deltaKO and C57BL/6 infected mice showed significant suppression at more than one concentration of ConA (Fig. 1).

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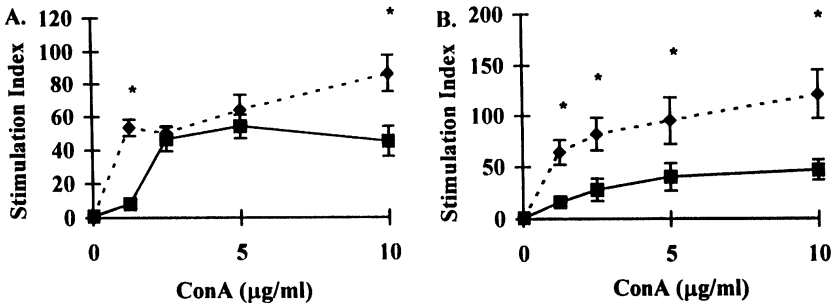

Fig. 1. Decreased mitogen-stimulated proliferation by spleen cells from heavily infected mice. Spleen cells from mice 52 days p.i. (■—■) and uninfected controls (◆---◆) were stimulated with ConA to evaluate responsiveness. Infected C57BL/6 mice (A) and deltaKO (B) mice showed suppression at more than one concentration of ConA. Error bars indicate ±S.E. (* indicates the value for infected mice is significantly different from the value for uninfected mice, $P < 0{\cdot}05$).

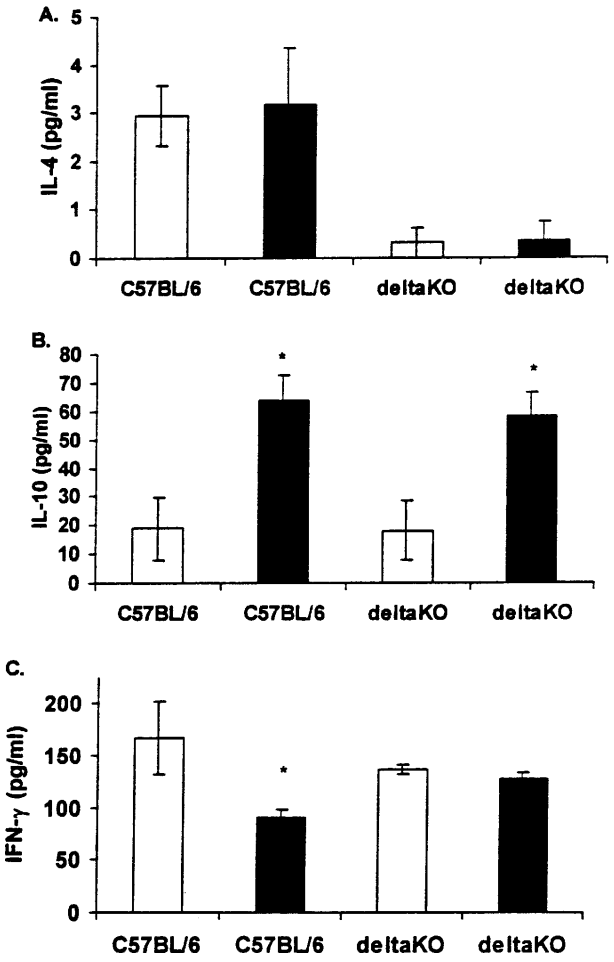

Fig. 2. ConA-stimulated cytokine production by cells from infected mice. Spleen cells of mice 52 days p.i. (■) and uninfected controls (□) were stimulated for 72 h with 5 μg/ml ConA. Supernatant fluids were collected and concentrations of IL-4 (A), IL-10 (B), and IFN-γ (C) were measured by sandwich ELISA. Error bars represent ± S.E. (* indicates the value for infected mice is significantly different from the value for uninfected mice, $P < 0{\cdot}05$).

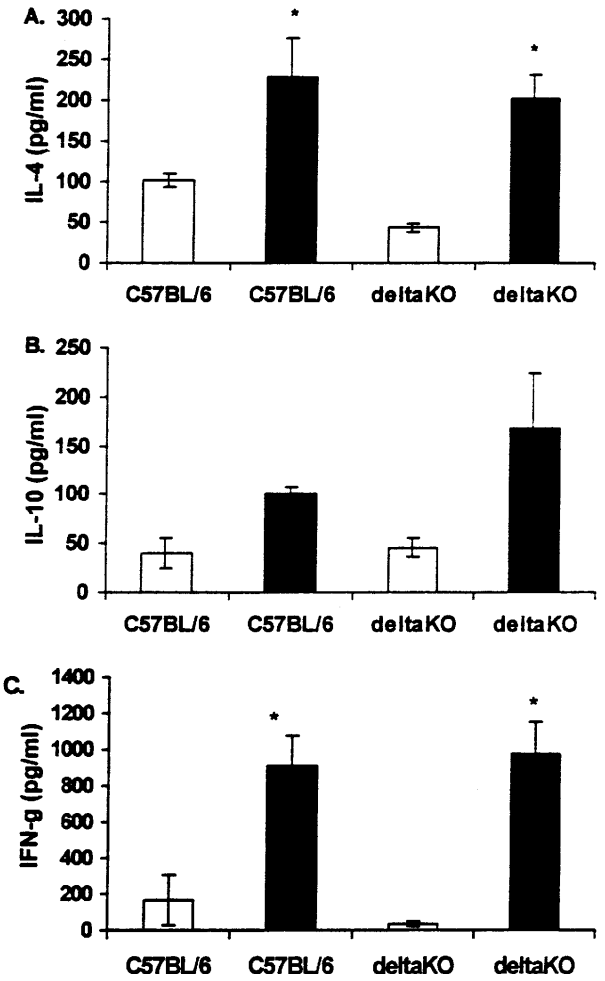

Fig. 3. Cytokine levels in serum from infected mice. Infected (■) and uninfected (□) mice were bled at 52 days p.i. and the concentrations of IL-4 (A), IL-10 (B), and IFN-γ (C) in serum were determined by sandwich ELISA. Error bars represent ± S.E. (* indicates the value for infected mice is significantly different from the value for uninfected mice, $P < 0{\cdot}05$).

ConA-stimulated cytokine production

Examination of splenic cytokine responses in infected C57BL/6 mice and deltaKO mice revealed no differences in IL-4 production between infected and uninfected C57BL/6 or deltaKO mice (Fig. 2A). All heavily infected mice (mice 52 days p.i.) showed a significant increase in IL-10 production when com-

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Table 1. Spleen cell percentages for normal and day 52-infected mice

(Data represent means ± S.D. of triplicate determinations.)

	Cell types		
Mouse strain	$\alpha\beta$TCR$^+$CD4$^+$	$\gamma\delta$TCR$^+$	CD8$^+$
Uninfected mice			
C57BL/6‡	19·01 ± 1·6	0·36 ± 0·19	12·64 ± 0·89
DeltaKO†	19·82 ± 0·2	0·0 ± 0·29	13·27 ± 0·6
Infected Mice			
C57BL/6§	20·71 ± 0·37*	0·70 ± 0·13	13·06 ± 0·94
DeltaKO§	21·00 ± 0·76*	0·10 ± 0·30	13·34 ± 1·54

* Indicates significant difference between normal and infected mice ($P < 0·05$).
† 3 mice per group.
‡ 5 mice per group.
§ 4 mice per group.

pared to uninfected controls (Fig. 2B). Thus, both strains of infected mice demonstrated an increased IL-10 production, while IFN-γ production was only decreased in C57BL/6 mice, but not deltaKO mice, and IL-4 production was essentially unaffected in these mice. This suggests that parasite growth is not inhibited in an immune environment where IL-10 production is increased in the spleen, regardless of whether IL-4 production is increased or IFN-γ production is decreased (deltaKO).

Lymphocyte subpopulations in spleens of infected mice

To investigate systemic immune responses of infected C57BL/6 and deltaKO mice serum cytokine levels were determined. Serum concentrations for uninfected mice agreed with values reported by other researchers (Ohkawara *et al*. 1997). Serum from infected mice showed increased concentrations of both IL-4 and IFN-γ (Fig. 3). Serum IL-10 was low but consistently elevated in infected deltaKO and C57BL/6 mice.

Spleen cell percentages in infected mice

Spleen cells of infected and uninfected C57BL/6 mice, and deltaKO mice were stained for analysis by flow cytometry to determine whether or not percentages of subpopulations of cells change during infection (Table 1). Infected C57BL/6 mice and deltaKO mice showed a slight increase in the percentage of $\alpha\beta$TCR$^+$CD4$^+$ cells. $\gamma\delta$TCR$^+$ cells were not detectable above background levels for deltaKO mice (as expected) and infection did not affect the percentage of $\gamma\delta$TCR$^+$ or CD8$^+$ cells in the spleens of the mice.

DISCUSSION

Infection of BALB/c mice with larvae of *T. crassiceps* induces a Th2-type response in splenocytes (Villa & Kuhn, 1996; Terrazas *et al*. 1998), and a mixed systemic Th1/Th2-type response in chronically-infected mice (Toenjes *et al*. 1999). Early in infection a Th1-type response is found in the spleen along with very little parasite growth (Terrazas *et al*. 1998). Moreover, delayed-type hypersensitivity-inducing drugs decrease the number of larvae obtained from infected mice (Bojalil *et al*. 1993). Thus, it seems that a Th1-type response may be successful in controlling larval growth. Interestingly, we have found that IL-10 production by peritoneal exudate cells (at the site of infection) is increased early in the immune response of BALB/cJ mice and, since an increase in IL-10 can be detected in the serum, this early increase in IL-10 acts systemically (Toenjes *et al*. 1999). It seems, therefore, that initially there may be a predominantly Th1-type response that is inhibited by the systemic effect of IL-10 resulting in non-protective responses.

In comparison to BALB/c mice, C57BL/6 mice have been reported to be relatively resistant to infection with larvae (Sciutto *et al*. 1995; Fragoso *et al*. 1996, 1998). Because $\gamma\delta$T cells are expanded in the body cavities of infected BALB/cJ mice (Toenjes *et al*. 1999), and $\gamma\delta$T cells are a known source of IFN-γ (Roberts, Ordway & Orme, 1993; Skeen & Ziegler, 1995; Hsieh *et al*. 1996; Duhindan *et al*. 1997), larval infection of δ-chain knock-out C57BL/6 (deltaKO) and normal C57BL/6 mice was investigated. It was found that C57BL/6 mice and deltaKO mice have similar parasite burdens at 52 days p.i. DeltaKO and C57BL/6 mice also have similar numbers of larvae at 100 and 150 days p.i., when the parasite burden is in the thousands (data not shown). Therefore, the equal parasite loads seen at 52 days p.i. are not due to the parasite being limited by factors such as nutrient availability. Thus, it seems that the presence of $\gamma\delta$T cells is not required in immunity to larvae. However, experiments with genetically altered knock-out mice should be carefully interpreted because redundancy in the immune system may mask the role that $\gamma\delta$T cells play in normal infections (Kaufmann & Ladel, 1994). For example, in listeriosis of β2-microglobulin knock-out mice, $\gamma\delta$T cells play a compensatory role in the absence of CD8$^+$ T cells, since depletion of $\gamma\delta$T cells in infected, normally resistant, β2-microglobulin knock-out mice results in susceptibility (Roberts *et al*. 1993).

To determine whether or not infected mice devoid of $\gamma\delta$T cells demonstrate immunosuppression, we investigated responsiveness of spleen cells of C57BL/6 and deltaKO mice to ConA stimulation. Because all infected mice showed suppression in mitogen-stimulated proliferation assays, these data

suggest that infection suppresses immune responses if worm burden reaches a critical level. Also, the percentages of T cells remained fairly constant in infected mice, suggesting that suppression of cells from heavily-infected mice is not simply due to a change in the types of cells present. Moreover, deltaKO mice demonstrate suppression greater than that for equally infected, normal C57BL/6 mice. Thus, γδT cells are not responsible for the suppression seen in the normal infection.

To investigate the cytokine profiles of splenic T cells and the systemic immune response of infected deltaKO and C57BL/6 mice, ConA-stimulated cytokine production and serum cytokine levels, respectively, were determined. Spleen cells from heavily infected C57BL/6 mice show an increase in ConA-stimulated IL-10 production with a decrease in IFN-γ production, both changes being indicative of a Th2-type response (Mosmann & Coffman, 1989), but no change in IL-4 production was found for C57BL/6 mice. Also, infected C57BL/6 mice showed increases in serum IL-4 and IFN-γ. DeltaKO mice showed similar cytokine profiles when compared to normal C57BL/6 mice; however, spleen cells from infected deltaKO mice do not show a decreased IFN-γ production in response to ConA.

These cytokine analyses suggest several things. First, based on the Th1-inhibitory actions of IL-10 (Fiorentino *et al*. 1991; Malefyt *et al*. 1991; Seder *et al*. 1992; Heinzel *et al*. 1993; Hsieh *et al*. 1993), spleen cells of chronically infected mice act to suppress the development of Th1-type responses. In addition, it seems that the presence of γδT cells is required for the decreased production of IFN-γ by spleen cells from infected C57BL/6 mice. Secondly, larval growth is not significantly affected in mice whose spleen cells do not show an increase in IL-4 production (C57BL/6 and deltaKO) or a decrease in IFN-γ production (deltaKO). Thus, increased spleen cell production of IL-10 best correlates with a non-protective immune response. Thirdly, the systemic immune response of all chronically infected mice tested cannot be classified as a typical Th1- or Th2-type response, and the presence of γδT cells is not required for the increases in serum IFN-γ and IL-4 found in chronically infected mice.

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REFERENCES

BOJALIL, R., TERRAZAS, L. I., GOVEZENSKY, T., SCIUTTO, E. & LARRALDE, C. (1993). Thymus-related cellular immune mechanisms in sex associated resistance to experimental murine cysticercosis (*Taenia crassiceps*). *Journal of Parasitology* **79**, 384–389.

DUHINDAN, N., FARLEY, A. J., HUMPHREYS, S., PARKER, C., ROSSITER, B. & BROOKS, C. G. (1997). Patterns of lymphokine secretion amongst mouse γδT cell clones. *European Journal of Immunology* **27**, 1704–1712.

FIORENTINO, D. F., ZLOTNIK, A., MOSMANN, T. R., HOWARD, M. & O'GARRA, A. (1991). IL-10 inhibits cytokine production by activated macrophages. *Journal of Immunology* **147**, 3815–3822.

FRAGOSO, G., LAMOYI, E., MELLOR, A., LOMELI, C., GOVEZENSKY, T. & SCIUTTO, E. (1996). Genetic control of susceptibility to *Taenia crassiceps* cysticercosis. *Parasitology* **112**, 119–124.

FRAGOSO, G., LAMOYI, E., MELLOR, A., LOMELI, C., HERNANDEZ, M. & SCIUTTO, E. (1998). Increased resistance to *Taenia crassiceps* murine cysticercosis in Qa-2 transgenic mice. *Infection and Immunity* **66**, 760–764.

FREEMAN, R. (1962). Studies on the biology of *Taenia crassiceps* (Zender 1980) Rudolphi, 1810 (Cestoda). *Canadian Journal of Zoology* **40**, 969–990.

GRAVETTER, F. J. & NALLUAU, L. B. (1996). *Statistics for the Behavioral Sciences*, 4th Edn., pp. 404–405, West Publishing Co., St Paul, MN.

HEINZEL, F. P., SCHOENHAUT, D. S., RERKO, R M., ROSSER, L. E. & GATELY, M. K. (1993). Recombinant interleukin-12 cures mice infected with *Leishmania major*. *Journal of Experimental Medicine* **177**, 1505–1509.

HERMANEK, J. & PROKOPIC, J. (1989). Influence of thymic preparations on the result of experimental infection with *Taenia crassiceps* (Zeder, 1800) in ICR mice. *Folia Parasitologica* **36**, 331–340.

HSIEH, C. S., MACATONIA, S. E., TRIPP, C. S., WOLF, S. F., O'GARRA, A. & MURPHY, K. M. (1993). Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* **260**, 547–549.

HSIEH, B., SCHRENZEL, M. D., MULVANIA, T., LEPPER, H. D., DI MOLFETTO-LANDON, L. & FERRICK, D. A. (1996). *In vivo* cytokine production in murine listeriosis. *Journal of Immunology* **156**, 232–237.

KAUFMANN, S. H. & LADEL, C. H. (1994). Role of T cell subsets in immunity against intracellular bacteria: experimental infections of knock-out mice with *Listeria monocytogenes* and *Mycobacterium bovis* BCG. *Immunobiology* **191**, 509–519.

MOSMANN, T. R. & COFFMAN, R. L. (1989). Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* **7**, 145–173.

MOSMANN, T. R. & SAD, S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology Today* **17**, 138–146.

OHKAWARA, Y., LEI, X. F., STAMPFLI, M. R., MARSHALL, J. S., XING, Z. & JORDANA, M. (1997). Cytokine and eosinophil responses in the lung, peripheral blood, and bone marrow compartments in a murine model of allergen-induced airways inflammation. *American Journal of Respiratory Cell and Molecular Biology* **16**, 510–520.

PAUL, W. E. & SEDER, R. A. (1994). Lymphocyte responses and cytokines. *Cell* **76**, 241–251.

ROBERTS, A. D., ORDWAY, D. J. & ORME, E. M. (1993). *Listeria monocytogenes* infection in beta 2 microglobulin-deficient mice. *Infection and Immunity* **61**, 1113–1116.

SALLY, C.-Y., CHAU, J. & FREEMAN, R. S. (1976). Intraperitoneal passage of *Taenia crassiceps* in rats. *Journal of Parasitology* **62**, 837–839.

SCIUTTO, E., FRAGOSO, G., BACA, M., DE LA CRUZ, V., LEMUS, L. & LARRALDE, C. (1995). Depressed T-cell proliferation associated with susceptibility to experimental *Taenia crassiceps* infection. *Infection and Immunity* **63**, 2277–2281.

SEDER, R. A., PAUL, W. E., DAVIS, M. M & FAZEKAS DE ST. GROTH, B. (1992). The presence of interleukin-4 during *in vitro* priming determines the lymphokine-producing potential of $CD4^+$ T cells from T cell receptor transgenic mice. *Journal of Experimental Medicine* **176**, 1091–1098.

SKEEN, M. J. & ZIEGLER, H. K. (1995). Activation of $\gamma\delta$T cells for production of IFN-γ is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *Journal of Immunology* **154**, 5832–5841.

TERRAZAS, L. I., BOJALIL, R., GOVEZENSKY, T. & LARRALDE, C. (1998). Shift from an early protective Th1 immune response to a late permissive Th2 response in murine cysticercosis (*Taenia crassiceps*). *Journal of Parasitology* **8**, 74–81.

TOENJES, S. A., SPOLSKI, R. J., MOONEY, R. A. & KUHN, R. E. (1999). The systemic immune response of BALB/c mice infected with larval *Taenia crassiceps* is a mixed Th1/Th2-type response. *Parasitology* **118**, 623–633.

VILLA, O. F. & KUHN, R. E. (1996). Mice infected with the larvae of *Taenia crassiceps* exhibit a Th2-like immune response with concomitant anergy and downregulation of Th1-associated phenomena. *Parasitology* **122**, 561–570.