J Trop Med Parasitol 2007;30:43-50.

ORIGINAL ARTICLE

Available online at www.ptat.thaigov.net

Expression Patterns of Intracellular Cytokines in Splenic Dendritic Cells from BALB/c Mice Infected with *Neospora caninum*

Punnarai Veeraseatakul[1, 2], Salakchit Chutipongvivate[1]

[1] Division of Clinical Pathology, Regional Medical Sciences Center, Department of Medical Sciences, Ministry of Public Health, Chiangmai 50180, Thailand
[2] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inadacho, Obihiro, Hokkaido 080-8555, Japan

Abstract

Using intracellular cytokine staining (ICCS) and flow cytometric analysis, the kinetics of cytokine expression were investigated in immunocompetent cells obtained from the spleens of non-infected and *Neospora caninum*-infected BALB/c mice, at 3, 5, and 7 days post-infection (dpi). The results showed that both Th1 and Th2 intracellular cytokines IL-4, IL-10, IL-12, and IFN-γ were detectable in both unfractionated spleen cells and splenic dendritic cells (DC) from non-infected and *N. caninum*-infected mice. IL-10 levels in the splenic DC of the infected mice were significantly up-regulated ($p < 0.05$) at 5 dpi. This expression declined rapidly by 7 dpi, followed by a concomitant increase in IL-12 expression. These results agree with the generally accepted concept that the balance between Th1 and Th2 cytokine response regulate the outcome of infection with pathogenic organisms, suggesting that they may play an important role in the resolution of infection.

Keywords: cytokine, dendritic cell, BALB/c mice, *Neospora caninum*

Introduction

Cytokines are chemical substances secreted by various immunocompetent cells, which are activated by the presence of antigen. The leukocytes that play an important role in the initial immune response include macrophages, neutrophils and dendritic cells (DC) [1,2]. Of these, DC represent a rare population of antigen-presenting cells (APC) in the blood, non-lymphoid and lymphoid tissues [3], linking the innate immune system with the adaptive specific immune system [4]. DC can produce cytokines, such as interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 15 (IL-15), interleukin 18 (IL-18), tumor necrosis factor-alpha (TNF-α), and chemokines [5]. Well-known studies of *Leishmania major* [6] and *Toxoplasma gondii* [2] have shown that DC can induce immune responses against parasites. *Neospora caninum* is an intracellular parasite, which was originally identified as a *T. gondii*-like parasite causing canine neuromuscular paralysis and bovine infertility and abortion [7]. IL-12 is essential for the stimulation of NK cells, differentiation of Th 1 cells and

Correspondence:
Punnarai Veeraseatakul, Regional Medical Sciences Center Chiangmai, 191 Moo 8, Donkaew, Maerim, Chiangmai 50180, Thailand
Tel: +66(0) 5311 2188; Fax: +66(0) 5311 2192
E-mail: punnarai@dmsc.moph.go.th

induction of IFN-γ production as a step toward mounting protection against *N. caninum* [8]. Khan *et al* [9] discovered that the spleen cells of infected A/J mice primarily produced significant quantities of IL-12, followed by IFN-γ at the early stage of infection, and these two cytokines were important in the natural protection against *N. caninum* infection. Moreover, mice treated with either anti-IL-12 or anti-IFN-γ antibody have been shown to succumb to a lethal infection similar to IFN-γ knock-out mice [10]. In other studies, the relative susceptibility of mice seems to be related to IL-4 levels, as resistance of B10.D_2 mice is associated with a lack of IL-4 production by splenocytes [8]. Also, the susceptibility of BALB/c mice corresponded to a high level of splenocyte IL-4 [11]. Nishikawa *et al* [12] detected significant levels of IFN-γ and IL-4 in the serum of resistant hosts, whereas IL-10 was detected in IFN-γ-deficient mice. Furthermore, TNF-α has been shown to exhibit a synergistic action with the IFN-γ production that inhibited *N. caninum* tachyzoite growth [13]. Intracellular cytokine expression patterns in DC following *N. caninum* infection have not yet been well elucidated; thus, the present study, aimed to clarify cytokine expression pattern in dendritic cells from the spleens of BALB/c mice during acute neosporosis, to better understand the DC function and host resistance to *N. caninum* infection.

Materials and methods

Mice and *N. caninum* infection

Inbred female, 4-6-week old, BALB/c mice were purchased from a commercial supplier (CLEA Japan Inc). All mice were kept in specific pathogen-free (SPF) conditions, with a 12:12 hour light-dark cycle, and were housed in cages (8 mice/cage) with free access to sterilized tap water and a standard commercial diet (Ca-1, CLEA Japan Inc). Twenty-four BALB/c mice were randomly divided into 3 groups of eight and infected intraperitoneally (ip) with 5×10^4 *N. caninum* tachyzoites in 0.1 ml PBS. The mice' spleens were collected at 3, 5, and 7 dpi for preparation of DCs and unfractionated splenocytes. Non-infected mice were sacrificed and used as controls.

Parasite culture and purification

N. caninum tachyzoites of the Nc-1 strain [7] were maintained in Vero cells in sterile tissue-culture medium; Minimum Essential Medium Eagle-MEM, SIGMA® supplemented with 8% heat-inactivated fetal bovine serum (FBS) (BroWhittacker, Maryland, USA) and 50 µg/ml kanamycin. Tachyzoites were purified from parasites and host cell debris following centrifugation in cold phosphate buffered saline (PBS). The resulting pellet was resuspended in cold PBS, passed through a 27-gauge needle and filtered using a 5.0 µm pore filter (Millipore, Bedford, Massachusetts, USA).

Antibodies

The anti-mouse monoclonal antibodies (mAbs) used for purification and staining were purchased from PharMingen (San Diego, USA); anti-IL-4 (clone 11B11), anti-IL-10 (clone JES5-16E3), anti-IL-12 (clone C15.6), anti-IFN-γ (clone XMG1.2) and anti-CD16/CD32 (clone 2.4G2). Anti-CD205 (clone NLDC-145) was purchased from Serotec (Oxford, England).

Isolation and purification of splenic dendritic cells

Single-cell suspensions from BALB/c mice spleens were isolated, as previously described by Makala and colleagues [14,15]. Briefly, immediately after sacrifice, the skin covering the abdomen was removed. Organs were removed aseptically, dissect away the serosa and fat, then placed in a glass Petri-dish with tissue culture medium, cut into tiny pieces and disrupted mechanically using a 5 ml syringe head and passage through a nylon cell strainer (70 µm-pore) (Becton Dickinson, New Jersey, USA). Contaminating red blood cells were lysed using 0.83% ammonium chloride and incubation for 5 min at 37°C. The cells were washed and resuspended in tissue culture medium to identify unfractionated splenocytes. For DC, the resulting cells were resuspended in 8 ml of tissue culture medium in a glass centrifuge tube and layered onto 2 ml of 14.5% w/v analytical grade metrizamide (Nycomed AS, Oslo, Norway) in RPMI

1640 without bicarbonate, supplemented with 2% FBS. The suspension was centrifuged at 600×g (with brakes off) for 10 min at room temperature. The low-density cells (LDC) were removed from the interface, washed twice and resuspended in PBS containing 3% FBS; these resident splenic DC were kept on ice until used.

Intracellular cytokine analysis staining (ICCS) using flow cytometry

The levels of *N. caninum* antigen-specific IL-4, IL-10, IL-12, and IFN-γ intracellular cytokine secretion and membrane surface antigens CD205 from splenic DC and unfractionated splenocytes were determined by flow cytometry, as described previously [16]. Briefly, cells were stained with fluorescein isothiocyanate (FITC) labeled anti-CD205 and anti-CD16/CD32 (Fcγ III/II receptor, FcBlockTM) at 4°C for 45 min, washed and resuspended in PBS/3% FBS, after which intracellular cytokine transport was blocked with brefeldin A (1 mg/ml) at 37°C for 4 hours, cells were fixed with 4% paraformaldehyde in PBS/0.02% EDTA at 37°C for 10 min, washed, and kept in PBS/0.02%EDTA/1%FCS overnight, or were frozen in 10% DMSO/90%FCS at -72°C until analysis. For intracellular cytokine staining, cells were permeabilized and stained in PBS/0.02%EDTA/1%FCS buffer containing 0.1% saponin. Binding was visualized by single-step technique, involving incubation with the following labeled anti-mouse mAbs: phycoerythrin (PE) labeled anti-IL-4, PE labeled anti-IL-10, PE labeled anti-IL-12 and PE labeled anti-IFN-γ. After incubation at 4°C for 45 min, the product was washed and resuspended with PBS. The cells (1×10^4) were collected and fluorescence was quantified using a Coulter EPICS-XL flow cytometer (Beckman Coulter, Miami, Florida, USA). Cells were electronically gated on forward-angle light scatter to exclude contaminating erythrocytes and small debris, and on 90" light scatter to exclude granulocytes.

Statistical analysis

The Student's t-test was used to analyze statistical data; $p < 0.05$ was considered statistically significant.

Results

Dendritic cell yield and purity

The cell yield from the spleens of 8 non-infected and *N. caninum*-infected BALB/c mice, isolated after mechanical disruption of spleen and ammonium chloride lysis but before metrizamide gradient centrifugation, ranged from 0.8×10^8 - 1.2×10^8 cells. After metrizamide density gradient centrifugation, the cell suspension consisted of about 0.9×10^7 - 1.2×10^7 cells. The isolated cells were characterized by high levels of dendritic cell marker CD205, compared with the unfractionated splenocytes before metrizamide density gradient centrifugation. The purity of the gradient cells, as determined by flow cytometry using CD205 expression, was always > 90% in both non-infected and *N. caninum*-infected mice (Table 1).

Table 1 Single-percentage expression of surface membrane marker CD205 on splenic dendritic cells (DCs), and unfractionated splenocytes from non-infected (day 0) and *N. caninum*-infected BALB/c mice. Data are expressed as mean percentages of three experiments ± standard deviation (± SD).

Specificity	Cell type	% Expression (Mean ± SD)			
		Day 0	3 dpi	5 dpi	7 dpi
CD205	splenic DC	90.8 ± 2.4	91.8 ± 5.4	93.0 ± 2.9	95.3 ± 1.3
	splenocyte	69.7 ± 2.0	77.8 ± 3.6	79.6 ± 9.8	87.6 ± 4.5

CD = cluster of differentiation; DCs = dendritic cells; dpi = days post-infection.

Expression of intracellular cytokines

The intracellular cytokine-mediated response to *Neospora* infection in BALB/c mice was evaluated. Flow cytometric analysis was used to determine the level of intracellular cytokine expression in splenic DC and unfractionated splenocytes obtained from non-infected and *N. caninum*-infected BALB/c mice, at 3, 5, and 7 dpi (Table 2; Figs 1, 2). The expression of IL-4, IL-10, IL-12, and IFN-γ was determined. As shown in Fig 1A, the expression of IL-4 in DC was stable throughout the study, whereas this expression increased rapidly in unfractionated splenocytes. IL-10 expression in the DC from the *N. caninum*-infected mice was up-regulated dramatically by 5 dpi, with a 10-fold increase over the non-infected controls. This expression declined rapidly 7 dpi. Meanwhile, there was an apparent 2-fold increase in this cytokine in unfractionated splenocytes 3-5 dpi; IL-10 expression, however, declined by 7 dpi (Fig 1B). A gradual decline in the expression of IL-12 from DC was observed 3-5 dpi, then up-regulated to higher levels than the control by 7 dpi. In contrast, a dramatic rise in IL-12 expression from unfractionated splenocytes 3 dpi, and a rapid decline by 5 and 7 dpi, were observed (Fig 1C). IFN-γ levels in DC decreased continuously throughout the study, while in unfractionated splenocytes, there was an increase by day 3 and a decline by 5-7 dpi; however, the levels remained above those of the control group (Fig 1D).

Discussion

This study aimed to assess cytokine expression patterns in immunocompetent cells from the spleens of non-infected and *N. caninum*-infected BALB/c mice at 3, 5, and 7 dpi, using ICCS and flow cytometric analysis. While BALB/c mice showed no clinical signs of neosporosis, both Th1 and Th2 intracellular cytokines were detectable at varying levels. ICCS with dual-color staining (FITC/PE) was used successfully to demonstrate exclusive or mutual co-expression of different cytokines and surface-membrane antigens: CD205 in spleen DC, thus allowing cell characterization by cytokine production and surface markers.

Table 2 Dual-percentage expression of intracellular cytokines on splenic dendritic cells (DC) and unfractionated splenocytes from non-infected (day 0) and *N. caninum*-infected BALB/c mice. Intracellular cytokine transport was blocked with brefeldin A, after which cells were fixed in paraformaldehyde, permeabilized with saponin and stained with FITC-labeled anti-CD205 and PE-labeled anti-IL-4, anti-IL-10, anti-IL-12, and anti-IFN-γ. Data are expressed as mean percentages of three experiments ± standard deviation (± SD).

Specificity	Cell type	% Expression (Mean ± SD)			
		Day 0	3 dpi	5 dpi	7 dpi
IL-4	splenic DC	1.4 ± 0.4	1.5 ± 0.1	1.7 ± 0.9	1.6 ± 0.2
	splenocyte	1.5 ± 0.2	5.2 ± 0.5	2.1 ± 0.3	3.3 ± 0.8
IL-10	splenic DC	3.7 ± 0.3	4.6 ± 1.0	32.5 ± 4.5	6.7 ± 0.6
	splenocyte	7.4 ± 0.3	14.5 ± 4.0	14.4 ± 2.4	5.8 ± 1.9
IL-12	splenic DC	4.9 ± 2.2	4.1 ± 1.7	2.3 ± 0.5	8.0 ± 2.2
	splenocyte	5.1 ± 1.5	7.9 ± 0.1	4.2 ± 1.5	3.1 ± 1.9
IFN- γ	splenic DC	2.8 ± 1.2	2.5 ± 0.9	2.1 ± 1.4	1.9 ± 0.5
	splenocyte	2.7 ± 0.4	6.1 ± 2.0	4.3 ± 1.4	3.9 ± 1.9

IL = interleukin, IFN-γ = interferon gamma, CD = cluster of differentiation, DC = dendritic cells, dpi = days post-infection.

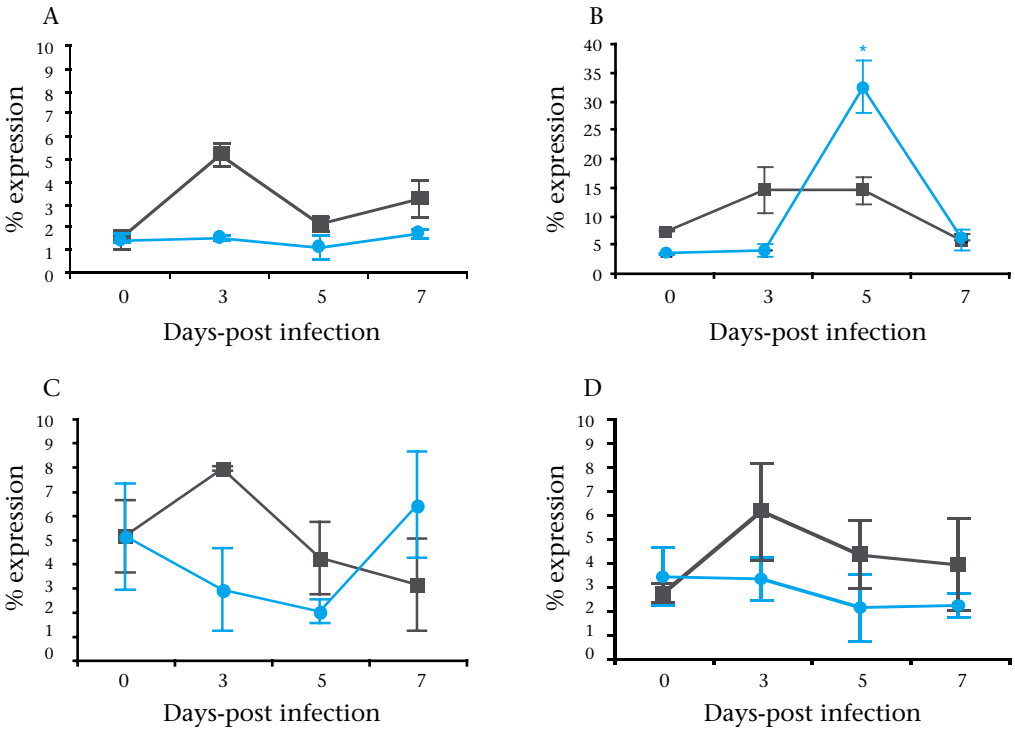

Fig 1 Patterns of intracellular cytokine expression of IL-4 (A), IL-10 (B), IL-12 (C), and IFN-γ (D) on splenic DC (●) and unfractionated splenocyte cells (■) from non-infected (day 0) and *N. caninum*-infected BALB/c mice at 3, 5, and 7 dpi. Intracellular cytokine transport was blocked with brefeldin A, after which cells were fixed in paraformaldehyde, permeabilized with saponin and stained with FITC-labeled anti-CD205 and PE-labeled anti-IL-4, anti-IL-10, anti-IL-12, and anti-IFN-γ. Data are expressed as mean percentages of three experiments ± standard deviation (± SD). (*) By Student's t-test, the differences between IL-10 expression in splenic DC from the infected mice on 5 dpi and the non-infected group were significant ($P < 0.05$).

We demonstrated that *N. caninum*, a coccidian parasite morphologically similar to *T. gondii*, can stimulate a post-infection immune response characterized by the production of immune cytokines, in particular, IL-10, IL-12, IL-4, and IFN-γ. IL-10 levels in splenic DC from *N. caninum*-infected mice were up-regulated to statistically significant levels, which peaked by 5 dpi and declined rapidly by 7 dpi. While IL-10 does not appear to be a required modulator at 7 dpi, the simultaneous induction of IL-10 and IFN-γ is a common feature of the immune response to intracellular parasites. IL-10 stimulation allows the pathogen to evade the IFN-γ-dependent mechanism of host resistance [17-19]. Our data demonstrated that the message for this cytokine is near above baseline at this time, corresponding with low cytokine production in response to parasitic infection. The ICCS data indicated that Th1 and Th2 cytokine balance may regulate the resolution of neosporosis infection.

The results of the current study were consistent with previous findings, which showed that IL-4, IFN-γ, and IL-12, compared with IL-10, showed significant delay in BALB/c mice [12]. All cytokines analyzed are essential for resistance

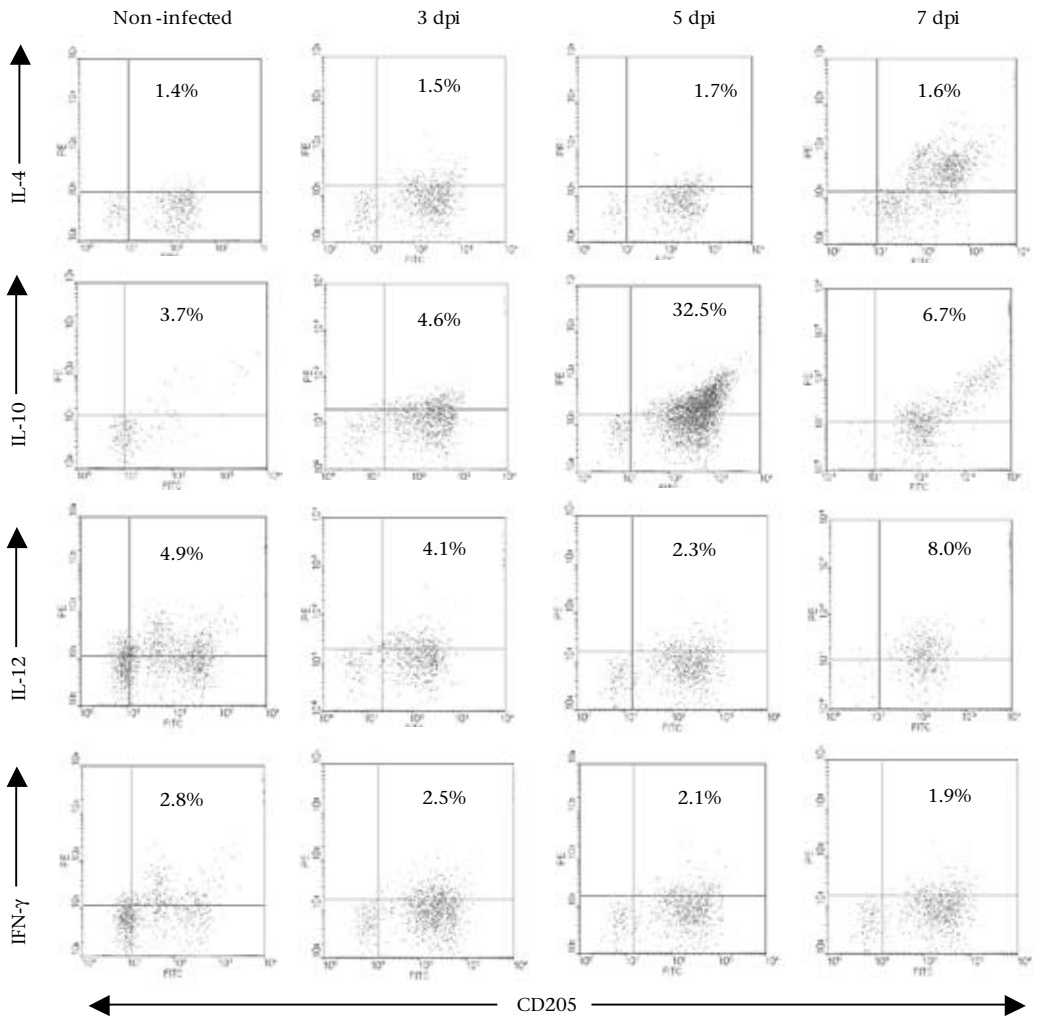

Fig 2 Intracellular cytokine expression patterns in splenic dendritic cells (DC) from non-infected and *N. caninum*-infected BALB/c mice at 3, 5, and 7 dpi. Intracellular cytokine transport was blocked with brefeldin A, after which cells were fixed in paraformaldehyde, permeabilized with saponin and stained with FITC-labeled anti- CD205 and PE-labeled anti-IL-4, anti-IL-10, anti-IL-12 and anti-IFN-γ. Percentages of positive dually stained-cells are given in the corresponding quadrants. The Y-axis is the log intensity of red (PE) fluorescence and the X-axis the log intensity of green (FITC) fluorescence. The data represent three experiments.

to *N. caninum* [8,9,18]. The mechanism of host resistance to *N. caninum* appears similar to the immune response elicited by *T. gondii*. Although the two parasites are genetically distinct and express different surface antigens, cross-reactive immunity between the organisms is apparent. This cross-reactivity can mostly easily be explained by the antigenic overlap between the immune epitopes. Interestingly, both parasites stimulate IL-12 production in the murine host [1,20]. It is likely that IL-10 may be responsible in part for loss of protection following depletion of IL-12. Further studies to elucidate the immune mechanism activated in response to *N. caninum* are needed.

Some studies have shown that resistance to neosporosis is associated with type 1 cytokine–IFN-γ and IL-12 [8,9], while susceptibility to *N. caninum* is associated with type 2 cytokine–IL-4 [11,21]. Enhanced disease and increased IL-4 production are also associated with other intracellular pathogens and bacterial infections. To date, correlative investigations have associated type-1 cytokines with resistance to *N. caninum*. In a study investigating various strains of mice, resistance to the development of disease was associated with the presence of IFN-γ and the absence of the type-2 cytokine, IL-4 [11]. The results of our study support the contention that *N. caninum* is a potent inducer of IL-4 in BALB/c mice and the presence of IL-4 during infection may determine the long-term outcome of disease [8]. The association of systemic IL-4 with *N. caninum*-induced disease was evident in earlier studies, which showed that encephalitis-resistant B10.D_2 mice had no IL-4 and high levels of IFN-γ, while encephalitis-susceptible C57BL/6 and BALB/c mice had high levels of both IL-4 and IFN-γ [11]. IL-4 has also been shown to cause termination of IL-12 signaling, through an initial IL-4 burst [22,23], and also interacts with IFN-γ at the effector level by competing for binding to IFN-interaction sequence motifs in antigen-presenting cells [24].

Whether or not the mechanisms of murine immune protection against *N. caninum* are active in other hosts that develop natural disease, such as cattle, horses, or dogs, or immunocompromised humans, is the big question. The present study indicates that future investigations of protective immunity against *N. caninum* infection should determine how to detect intracellular cytokines qualitatively by PCR technique.

Acknowledgements

We sincerely thank Dr Levi Makala for excellent help and support, and the National Research Center for Protozoan Disease for the work-place.

References

1. Gazzinelli RT, Hieny S, Wynn TA, Wolf S and Sher A. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc Natl Acad Sci. 1993;90:6115-9.
2. Reis e Sousa C, Hieny S, Scharton-Kersten T, Jankovic D, Charest H, Germain RN, *et al*. *In vivo* microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. J Exp Med. 1997;186:1819-29.
3. Makala LH, Reyes JC, Nishikawa Y, Tsushima Y, Xuan X, Huang X, *et al*. Phenotype and function of murine discrete Peyer's patch macrophage derived-dendritic cells. J Vet Med Sci. 2003a;65:491-9.
4. Makala LH, Nishikawa Y, Mishima M, Inoue N, Xuan X, Suzuki H, *et al*. Phenotype and function of murine peritoneal cavity macrophage derived-dendritic cells. J Vet Med Sci. 2002a;64:813-20.
5. Makala LH and Nagasawa H. Dendritic cells: a specialized complex system of antigen presenting cells. J Vet Med Sci. 2002b;64:181-93.
6. Blank C, Fuchs H, Rapperberger K, Rollinghoff M and Moll H. Parasitism of epidermal Langerhans cells in experimental cutaneous leishmaniasis with *Leishmania major*. J Infect Dis. 1993;167:418-25.
7. Dubey JP and Lindsay DS. Review of *Neospora caninum* and neosporosis. Vet Parasitol. 1996;67:1-59.
8. Baszler TV, Long MT, McElwain TF and Mathison BA. Interferon-gamma and interleukin-12 mediate protection to acute *Neospora caninum* infection in BALB/c mice. Int J Parasitol. 1999;29:1635-46.
9. Khan IA, Schwartzman JD, Fonseka S and Kasper LH. *Neospora caninum*: Role for immune cytokines in host immunity. Exp Parasitol. 1997;85:24-34.
10. Dubey JP, Hattel AL, Lindsay DS and Topper MJ. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J Am Vet Med

Assoc. 1998;193:1259-63.

11. Long MT, Baszler MT and Mathison BA. Comparison of intracerebral parasite load, lesion development and systemic cytokines in mouse strains infected with *Neospora caninum*. J Parasitol. 1998;84:316-20.
12. Nishikawa Y, Tragoolpua K, Inoue N, Makala L, Nagasawa H, Otsuka H, *et al*. In the absence of endogenous gamma interferon, mice acutely infected with *Neospora caninum* succumb to a lethal immune response characterized by inactivation of peritoneal macrophages. Clin Diag Lab Immunol. 2001;8:811-16.
13. Yamane I, Kitani H, Kokuho T, Shibahara T, Haritani M, Hamaoka T, *et al*. The inhibitory effect of interferon gamma and tumor necrosis factor alpha on intracellular multiplication of *Neospora caninum* in primary bovine brain cells. J Vet Med Sci. 2000;62:347-51.
14. Makala LHC, Haverson K, Stokes CR, Bailey M and Bland PW. Isolation and characterization of pig Peyer's patch dendritic cells. Vet Immunol Immunopathol. 1998;61:67-81.
15. Makala LHC, Reyes JCS, Nishikawa Y, Tsushima Y, Xuan X, Huang X, *et al*. A comparison of the phenotype of dendritic cells derived from discrete Peyer's patch macrophages of non-infected and *Toxoplasma gondii* infected mice. J Vet Med Sci. 2003b;65:591-7.
16. Huang LY, Reis e Sousa C, Itoh Y, Inman J and Scott DE. IL-12 induction by a Th1-inducing adjuvant *in vivo*: dendritic cell subsets and regulation by IL-10. J Immunol. 2001;167:1423-30.
17. Denis M and Ghadirian E. IL-10 neutralization augments mouse resistance to systemic *Mycobacterium avium* infections. J Immunol. 1993;151:5425-30.
18. Khan IA, Matsura T and Kasper LH. IL-10 mediates immunosuppression following primary infection with *Toxoplasma gondii* in mice. Parasite Immunol. 1995;17:185-95.
19. Sher A, Fiorentino D, Caspar P, Pearce E and Mosmann T. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. J Immunol. 1991;147:2713-6.
20. Khan IA, Matsuura T and Kasper LH. Interleukin-12 enhances murine survival against acute toxoplasmosis. Infect Immun. 1994;62:1639-42.
21. Long MT and Baszler TV. Neutralization of maternal IL-4 modulates congenital protozoal transmission: comparison of innate versus acquired immune responses. J Immunol. 2000;164:4768-74.
22. Launois P, Swihart KG, Milon G and Louis JA. Early production of IL-4 in susceptible mice infected with *Leishmania major* rapidly induces IL-12 unresponsiveness. J Immunol. 1997;158:3317-24.
23. Himmelrich H, Parra-Lopez C, Tacchini-Cottier F, Louis JA and Launois P. The IL-4 rapidly produced in BALB/c mice after infection with *Leishmania major* down-regulates IL-12 receptor beta 2-chain expression on CD4+ T cells resulting in a state of unresponsiveness to IL-12. J Immunol. 1998;161:6156-63.
24. Ohmori Y and Hamilton TA. IL-4-induced STAT6 suppresses IFN-gamma-stimulated STAT1-dependent transcription in mouse macrophages. J Immunol. 1997;159:5474-82.