In vitro lymphoproliferative response and cytokine production in mice with experimental disseminated candidiasis

Ali Reza Khosravi 1*, Hojjatollah Shokri 2, Shahin Eshghi 3

1 Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
2 Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
3 Department of Food Sciences, Iranian Veterinary Organization, Qazvin, Iran

ARTICLE INFO

Article type:
Original article

Article history:
Received: Dec 8, 2015
Accepted: Apr 28, 2016

Keywords:
Candida albicans
Cytokine production
Lymphocyte proliferation
Mitogens
Mice
Systemic candidiasis

ABSTRACT

Objective(s): Systemic candidiasis is an infection of Candida albicans (C. albicans) causing disseminated disease and sepsis, invariably when host defenses are compromised. We investigated the histopathological changes as well as the lymphoproliferative responses and cytokine production of splenic cells after stimulation with Concanavalin A (Con A) and Pokeweed mitogen (PWM) in mice with disseminated candidiasis.

Materials and Methods: Lymphoproliferative responses were stimulated in vitro with Con A (1 µg/ml) and PWM (1 µg/ml) mitogens in Roswell Park Memorial Institute (RPMI) 1640 media, and the production of interferon (IFN)-γ and interleukin-4 (IL-4) in the supernatants was measured by enzyme-linked immunosorbent assay (ELISA).

Results: The results revealed that C. albicans organisms multiplied to a greater extent in the kidneys than in the liver and spleen of infected mice. The most predominant forms of C. albicans in different parts of the kidneys were yeast mixed with hyphal forms. Infected mice had a significantly increased proliferative response when splenocytes were stimulated with PWM (2.0±0.16) and Con A (1.9±0.19) (P<0.05). PWM and Con A-stimulated production of IFN-γ significantly tended to be higher in infected mice (PWM: 68.4±14.0 pg/ml; Con A: 53.7±17.3 pg/ml) when compared to controls (P<0.05). Stimulation with PWM and Con A showed no differences in IL-4 production between infected mice and controls.

Conclusion: These findings demonstrated a significant increase in both cell proliferation and IFN-γ secretion in supernatants of PWM and Con A-stimulated splenocyte cultures obtained from mice with disseminated candidiasis.

Introducction

Candida infection became recognised as being of clinical importance over the past two to three decades due to a combination of rising numbers of patients with immunodeficiency illnesses and improvements in general life expectancies. The nature and extent of the impairment of the host defense influence the manifestation and severity of infection (1, 2). The innate and adaptive immune responses are required for development of resistance to disseminated candidiasis. Neutrophils and macrophages can clear the pathogen via phagocytosis, and macrophage activation also leads to the release of several key mediators such as pro-inflammatory cytokines, which are important for protecting the host against disseminated candidiasis (3, 4). In candidiasis, the initial handling of fungal pathogen by cells of the innate immune system plays a major role in determining CD4+ T helper (Th) development.

Th1 and Th2 CD4+ T-cells have been shown to influence the pathway of differentiation of CD4+ T-cell precursors (5). The different cytokine patterns lead to different functions, such as the modulation of antifungal effector activity and the regulation of various Th subsets development. Previous studies demonstrated that the development of protective Th1 responses in mice with disseminated candidiasis requires the coordinated functions of several cytokines such as, interferon (IFN)-γ (6), transforming growth factor (TGF)-β (7), interleukin (IL)-6 (8), tumor necrosis factor (TNF)-α (9) and IL-12 (10), in the relative absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit the development of Th1 responses (11). The neutralization of Th1 cytokines (IFN-γ and IL-12) in early infection results in immune responses towards the Th2 instead of Th1 cells, whereas neutralization of Th2 cytokines (IL-4 and IL-10) is responsible for a shift of Th2 towards the Th1 responses (12). Thus, Th1 mediates anti-Candida albicans host defense through the production of IFN-γ, which is required for optimal activation of phagocytes.

*Corresponding author: Ali Reza Khosravi. Mycology Research Center, Faculty of Veterinary Medicine, Tehran, Iran. Tel: +98-21-61117151; Fax: +98-21-66933222; email: khosravi@ut.ac.ir

d cytokine production of
hosravi. Mycology Research Center, Faculty of Veterinary Medicine, Tehran, Iran. Tel: +98-21-66933222

M (1 µg/ml) mitogens in Roswell Park Memorial Institute (RPMI)
s the
nd IL-4. In candidiasis, the initial handling of fungal pathogen
protecting the host against disseminated candidiasis (3, 2). The
impairment of the hos
general life expectancies. The nature and extent of the
immunodeficiency illnesses and improvements in

Introduction

Candida infection became recognised as being of clinical importance over the past two to three decades due to a combination of rising numbers of patients with immunodeficiency illnesses and improvements in general life expectancies. The nature and extent of the impairment of the host defense influence the manifestation and severity of infection (1, 2). The innate and adaptive immune responses are required for development of resistance to disseminated candidiasis. Neutrophils and macrophages can clear the pathogen via phagocytosis, and macrophage activation also leads to the release of several key mediators such as pro-inflammatory cytokines, which are important for protecting the host against disseminated candidiasis (3, 4). In candidiasis, the initial handling of fungal pathogen by cells of the innate immune system plays a major role in determining CD4+ T helper (Th) development.

Th1 and Th2 CD4+ T-cells have been shown to influence the pathway of differentiation of CD4+ T-cell precursors (5). The different cytokine patterns lead to different functions, such as the modulation of antifungal effector activity and the regulation of various Th subsets development. Previous studies demonstrated that the development of protective Th1 responses in mice with disseminated candidiasis requires the coordinated functions of several cytokines such as, interferon (IFN)-γ (6), transforming growth factor (TGF)-β (7), interleukin (IL)-6 (8), tumor necrosis factor (TNF)-α (9) and IL-12 (10), in the relative absence of inhibitory Th2 cytokines, such as IL-4 and IL-10, which inhibit the development of Th1 responses (11). The neutralization of Th1 cytokines (IFN-γ and IL-12) in early infection results in immune responses towards the Th2 instead of Th1 cells, whereas neutralization of Th2 cytokines (IL-4 and IL-10) is responsible for a shift of Th2 towards the Th1 responses (12). Thus, Th1 mediates anti-Candida albicans host defense through the production of IFN-γ, which is required for optimal activation of phagocytes.

*Corresponding author: Ali Reza Khosravi. Mycology Research Center, Faculty of Veterinary Medicine, Tehran, Iran. Tel: +98-21-61117151; Fax: +98-21-66933222; email: khosravi@ut.ac.ir
And for helping in the generation of a protective antibody response. Altogether, these data demonstrate that resistance or susceptibility to infections correlates with the levels of Candida growth in target organs, as well as with the type of Th cytokine production by specific CD4+ T lymphocytes. It has been hypothesized that a defective Th1 response may be at least partially responsible for the persistence of Candida infection in patients with disseminated candidiasis. The aims of this study were to investigate the histopathological changes as well as the lymphoproliferative responses and cytokine production of splenic cells after stimulation with Concanavalin A (Con A) and Pokeweed mitogen (PWM) in mice with disseminated candidiasis.

Materials and Methods

Animal model
Female BALB/c mice, 21 days of age, were purchased from the Razi Institute, Karaj in Iran. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Tehran, and were carried out in accordance with the National Health and Medical Research Council’s Iranian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice were housed in standard cages, and provided with food and water ad libitum.

Yeast
C. albicans ATCC 10231 was obtained from the Mycology Research Center (MRC), University of Tehran, Tehran in Iran. The yeast cells were cultured in Sabouraud dextrose agar (SDA) (Merck Co, Darmstadt, Germany) at 37 °C for 20 hr. Then the blastospores were collected by centrifugation, washed twice with phosphate-buffered saline (PBS), and adjusted to 2×10^6 blastospores per ml in PBS until use (13).

Infection model
Mice were divided into two groups including infected group (15 animals) and control group (10 animals). Infected group was mice infected with C. albicans [intravenously (IV), 2×10^6 cell/ml, 0.2 ml] and control group was mice received normal saline (NaCl 0.9 %) (IV, 0.2 ml/day).

Histopathology
At 7 days post-infection, 5 infected mice were killed and the kidneys, spleen and liver were removed aseptically, weighed and homogenized in 1 ml PBS; dilutions of the homogenates were cultured onto SDA. The Colony forming units (CFUs) were counted after 24 hr of incubation at 37 °C and expressed as CFU per gram (13).

Preparation of splenocytes
Spleens were removed from 10 infected mice and 10 control mice, and spleen cells were obtained by pressing through a sterile metal sieve, followed by filtration through an 80-mm nylon mesh. The cells were resuspended in 6 ml of PBS, and lymphocytes were separated on a Ficoll gradient by underlaying the cell suspension with 4 ml Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). The gradient was centrifuged at 340 g for 10 min at room temperature. The buffy coat interface was carefully removed, washed twice in PBS, and resuspended in 1 ml of PBS. Viable lymphocytes were counted in a haemocytometer after staining with trypan blue (Sigma, St Louis, Mo, USA) (14).

Mitogens proliferation
Mitogen proliferation assays were performed by incubating 10^5 splenic cells in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine and 20% fetal calf serum (FCS). The cells were cultured in triplicate for 72 hr with Con A (1 µg/ml) (Sigma, St. Louis, Mo, USA) and PWM (1 µg/ml) (Sigma, St. Louis, Mo, USA) at 37 °C in 5% CO2. After 72 hr, they received a 6-hr pulse with 0.5 mCi of [3H]thymidine (Sigma, St Louis, Mo, USA) and were then harvested and washed on glass filters. [3H]thymidine incorporation was measured in a liquid scintillation counter (Beckman, Palo Alto, CA, USA). The results were presented as stimulation index (SI) (15).

Cytokines assay
A) IFN-γ assay
The amount of IFN-γ present in the supernatant at 72 hr of culture was determined by using a mouse IFN-γ ELISA kit (BioSource International, Camarillo, Calif., USA). Briefly, 100 ml of supernatant or mouse IFN-γ standard was added in duplicate to each well of a plate precoated with antimouse IFN-γ monoclonal antibody, and the plate was incubated for 1 hr at 24 °C. The plate was then washed once with a buffered detergent wash solution, 100 ml of a rabbit polyclonal anti-mouse IFN-γ antibody was added to each well, and the plate was incubated for 1 hr at 24 °C. The plate was washed, 100 ml of an anti-rabbit antibody conjugated to horseradish peroxidase (HRP) was added to each well, and the plate was incubated for 1 hr at 24 °C. The plate was washed 4 times, an HRP substrate solution was added for 1 hr. The reaction was stopped by adding 5% sulfuric acid to each well, and the plate was read at 450 nm. The values were calculated by comparison with the standard curve.

B) IL-4 assay
IL-4 was measured in cell culture supernatants at 48 hr by using an IL-4 ELISA kit (Genzyme, Cambridge, MA, USA). A 96-well plate was coated with mouse monoclonal anti-mouse IL-4 and incubated at 4 °C overnight. The plate was then washed with a PBS–Tween solution, the IL-4 standards and supernatants were applied to the plate, and the plate was incubated for 2 hr at room temperature. After washing, 100 ml of polyvalent rabbit anti-mouse IL-4 was added to each well and the plate was again incubated for 2 hr at room temperature. The plate was washed, biotin-conjugated goat anti-rabbit immunoglobulin was added, and the
Systemic candidiasis and mitogens

Khosravi et al

Iran J Basic Med Sci, Vol. 20, No. 2, Feb 2017

195

Figure 1. Mean weight of different mice groups at day 7 - d1, d2 and d3: days 1, 2 and 3, respectively

Plate was incubated for 45 min. After washing 100 ml of streptavidin-conjugated HRP was added to each well for 40 min. The plate was washed one more time. The substrate reagent (chromogen with peroxidase) was then added to each well. The reaction was allowed to proceed for 10 min and was then stopped by the addition of 1 M H₂SO₄ to each well; the A 450 was read, and the values were calculated from the standard curve.

Statistical analysis
Statistical evaluation was performed by ANOVA test. Values were considered significant at P<0.05.

Results
The results showed that the viable yeast cells in the kidneys reached approximately 5.6 log10 CFU/g at day 7 post-infection, followed by the spleen (3.9 log10 CFU/g) and liver (3.7 log10 CFU/g). The infected mice group had lower weight than control group at days 1, 3 and 7 after infection (Figure 1). No significant differences were observed between infected and control mice groups at different days after infection (P=0.089). As shown in Figure 2, the kidneys were the organs with the highest burdens of C. albicans throughout the observation period. The kidneys revealed vascular congestion, haemorrhages, tubular degeneration and heterophilic infiltration. The glomeruli showed hypercellularity and glomerular tufts were atrophied in places revealing an expanded Bowman’s capsule. The medulla revealed multiple necrotic foci and an inflammatory reaction initially predominated by neutrophils. The renal papillae and pelvis showed extensive necrosis with infiltrating and macrophages. Fungal elements demonstrated within tissues with Gomori Methanamine Silver (GMS) stain consisted of chains of elongate yeast-like structures and tubular, septate, and branched hyphae.

In infected mice, all animals presented significantly more proliferative responses to PWM (2.0±0.16) and Con A (1.9±0.19) when compared to control mice stimulated with PWM (1.7±0.19) and Con A (1.6±0.15) mitogens (P<0.05). The median values demonstrated a clear mitogen stimulatory effect in the following order: PWM > Con A > cell control (Figure 3).

As illustrated in Figure 4, lymphocytes from mice with disseminated candidiasis produced significantly more IFN-γ in response to PWM (68.4±14.0 pg/ml) than controls (PWM: 35.1±9.3 pg/ml) (P<0.05). In addition, the mean level of production of IFN-γ after stimulation with Con A was significantly different between infected mice (53.7±17.3 pg/ml) and control (28.9±11.3 pg/ml) groups (P<0.05).

Figure 3. Proliferative response of mononuclear cells from infected and control mice under different stimuli

Figure 4. IFN-γ production by stimulated mononuclear cells in culture
mononuclear cells in infected and control groups, respectively. The differences in infection between the colonization patterns of yeast-like and an accumulation of lymphocytes. The differences in resistance to two morphological forms. The kidneys were the organs with the highest burdens of *C. albicans* throughout the observation period. They revealed vascular congestion, haemorrhages, tubular degeneration and heterophilic infiltration. Fungal elements consisted of chains of elongate yeast-like structures and tubular, septate, and branched hyphae. Khosravi *et al* (13) showed that both the yeast and hyphal forms of *C. albicans* are capable of invading soft tissues and the ability of tissue fluids to interconvert the two morphological forms.

The results of this study demonstrated that host resistance to *C. albicans* infection in a murine model is linked to a particular pattern of cytokine response and an accumulation of lymphocytes. The differences between the colonization patterns of *C. albicans* in "infection-resistant" BALB/c mice and "infection-prone" mice following infection correlated with both T-cell proliferation and the secretion pattern of the cytokines IL-4, IL-12, and IFN-γ (18). The lymphoproliferative responses were analyzed after stimulation with PWM and Con A mitogens. In infected mice, all animals presented significantly more proliferative responses to PWM (2.0±0.16) and Con A (1.9±0.19) when compared to control mice stimulated with PWM (1.7±0.19) and Con A (1.6±0.15) mitogens (*P*<0.05). The median values demonstrated a clear mitogen stimulatory effect in the following order: PWM > Con A > cell control. In consistence with our results, Katial *et al* (19) demonstrated that PWM was much more effective in proliferating lymphocytes than the other mitogens at a median value above the baseline (cell control) value.

The present study showed that lymphocytes from mice with disseminated candidiasis produced significantly more IFN-γ in response to PWM (68.4±14.0 pg/ml) than controls (PWM: 35.1±9.3 pg/ml) (*P*<0.05). In addition, the mean level of production of IFN-γ after stimulation with Con A was significantly different between infected mice (53.7±17.3 pg/ml) and control (28.9±11.3 pg/ml) groups (*P*<0.05). The increased levels of IFN-γ noted secondary to PWM may have been due to dual stimulation of both T and B lymphocytes. The B cells may have produced an amplification factor which then stimulated the T lymphocytes to increase IFN-γ production.

In primary disseminated candidiasis, IL-4 may limit *C. albicans* infection through promoting effector mediators of immunity, including the differentiation of effector Th1 cells. In particular, IL-4 promotes the development of a protective Th1 response in disseminated candidiasis (20). In our study, detectable levels of mitogens-stimulated IL-4 production were present in both infected and control mice groups. The mean values of IL-4 production induced by different mitogens: PWM-stimulated IL-4 production in infected mice resulted in levels of 5.6±1.9 pg/ml when compared to 8.8±2.0 pg/ml for controls, while values detected after Con A stimulation were 2.0±1.2 pg/ml and 2.6±1.1 pg/ml for infected and control groups, respectively (*P*=0.075).

### Discussion

Exploration of immunological events leading to *Candida* resistance or susceptibility has indicated the central role of the innate and adaptive immune systems, the relative contribution of each type of immune responses depends on the site of the primary infection. The results exhibited that viable yeast cells in the kidneys reached approximately 5.6 log_{10} CFU/g at day 7 post-infection, followed by the spleen (3.9 log_{10} CFU/g) and liver (3.7 log_{10} CFU/g). Our results are in consistent with other researchers (13, 16, 17) who reported *Candida* organisms multiplied to a greater extent in the kidneys than in the liver and spleen of animals. A correlation was made between the pathological lesions observed in the groups and the mean mice weight changes. Histopathologic examination revealed pyo-granulomatous inflammation with intraserial fungal organisms in several organs including the kidneys, brain, spleen and liver. The kidneys were the organs with the highest burdens of *C. albicans* throughout the observation period. They revealed vascular congestion, haemorrhages, tubular degeneration and heterophilic infiltration. Fungal elements consisted of chains of elongate yeast-like structures and tubular, septate, and branched hyphae. Khosravi *et al* (13) showed that both the yeast and hyphal forms of *C. albicans* are capable of invading soft tissues and the ability of tissue fluids to interconvert the two morphological forms.

The results of this study demonstrated that host resistance to *C. albicans* infection in a murine model is linked to a particular pattern of cytokine response and an accumulation of lymphocytes. The differences between the colonization patterns of *C. albicans* in "infection-resistant" BALB/c mice and "infection-prone" mice following infection correlated with both T-cell proliferation and the secretion pattern of the cytokines IL-4, IL-12, and IFN-γ (18). The lymphoproliferative responses were analyzed after stimulation with PWM and Con A mitogens. In infected mice, all animals presented significantly more proliferative responses to PWM (2.0±0.16) and Con A (1.9±0.19) when compared to control mice stimulated with PWM (1.7±0.19) and Con A (1.6±0.15) mitogens (*P*<0.05). The median values demonstrated a clear mitogen stimulatory effect in the following order: PWM > Con A > cell control. In consistence with our results, Katial *et al* (19) demonstrated that PWM was much more effective in proliferating lymphocytes than the other mitogens at a median value above the baseline (cell control) value.

The present study showed that lymphocytes from mice with disseminated candidiasis produced significantly more IFN-γ in response to PWM (68.4±14.0 pg/ml) than controls (PWM: 35.1±9.3 pg/ml) (*P*<0.05). In addition, the mean level of production of IFN-γ after stimulation with Con A was significantly different between infected mice (53.7±17.3 pg/ml) and control (28.9±11.3 pg/ml) groups (*P*<0.05). The increased levels of IFN-γ noted secondary to PWM may have been due to dual stimulation of both T and B lymphocytes. The B cells may have produced an amplification factor which then stimulated the T lymphocytes to increase IFN-γ production.

In primary disseminated candidiasis, IL-4 may limit *C. albicans* infection through promoting effector mediators of immunity, including the differentiation of effector Th1 cells. In particular, IL-4 promotes the development of a protective Th1 response in disseminated candidiasis (20). In our study, detectable levels of mitogens-stimulated IL-4 production were present in both infected and control mice groups. The mean values of IL-4 production induced by different mitogens: PWM-stimulated IL-4 production in infected mice resulted in levels of 5.6±1.9 pg/ml when compared to 8.8±2.0 pg/ml for controls, while values detected after Con A stimulation were 2.0±1.2 pg/ml and 2.6±1.1 pg/ml for infected and control groups, respectively (*P*=0.075). MITogens-stimulated responses, although low, were detectable. It has been shown that IL-4- producing cells in the periphery are scarce (21). In our study, the susceptibility of infected mice was lower than that of control mice in view of IL-4 production. In consistence with our results, Kaposzta *et al* (22) revealed low level of IL-4 in mice with disseminated candidiasis. In addition, other studies have shown that mice deficient in IL-4 were more susceptible to acute disseminated infection than normal controls, though no difference in susceptibility to disseminated candidiasis after challenge was noted (23). In contrast, Romani *et al*...
(24) demonstrated that neutralization of IL-4 or IL-4 receptor was associated with an increased resistance to C. albicans. These paradoxical findings may be explained by different experimental models, different mouse strains, different routes of challenge and doses of C. albicans to induce systemic candidiasis, the condition of the infected host and stage of infection. In addition, this difference could be explained by unrelated genetic factors, which influence the immune response and susceptibility of other inbred strains to the growing pathogen.

The explanation of why each mitogen stimulates the cells to produce different levels of cytokines is not clear, although the spectrum of target cells for each mitogen is known to be somewhat different. Perhaps mitogens studied were not potent stimulators for cytokines production, in particular IL-4. Con A is reported to stimulate cytotoxic T cells (25), suppressor inducer T cells (26), or "virgin" T cells (27). PWM, on the other hand, stimulates helper T cells and, in association, B cells (27). Our results showed that cytokine levels vary based on different mitogens.

Conclusion

Our study demonstrated a significant increase in both cell proliferation and IFN-γ secretion in PWM and Con A- stimulated splenocyte cultures from mice with disseminated candidiasis. The results presented above suggest that one needs to be very aware of the culture conditions, the mitogens used, and the clinical state of the animals when performing in vitro cytokine measurements.

Acknowledgment

This work was supported by the Research Council of the University of Tehran, Tehran, Iran. The results described in this paper were part of student thesis.

Conflict of interest

The authors declared no conflict of interest.

References

1. Calderone RA. Candida and Candidiasis. New York: American Society for Microbiology; 2001.
2. Garber G. An overview of fungal infections. Drugs 2001; 61:1-12.
3. Romani L, Mencacci A, Cenci E, Del Sero G, Bistoni F, Puccetti P. An immunoregulatory role for neutrophils in CD41 T helper subset selection in mice with candidiasis. J Immunol 1997; 158:2356-2362.
4. Vasquez-Torres A, Balish E. Macrophages in resistance to candidiasis. Microbiol Mol Rev 1997; 61:170-192.
5. O’Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immum 1998; 8:275-285.
6. Cenci E, Mencacci A, Del Sero G, Fed Ostiani C, Mosci P, Kopf M. IFN-γ is required for IL-12 responsiveness in mice with Candida albicans infection. J Immunol 1998; 161:3543-3550.
7. Chin VK, Foong KJ, Maha A, Rusilza B, Norhaziah M, Chong PP. Multi-step pathogenesis and induction of local immune response by systemic Candida albicans infection in an intravenous challenge mouse model. Int J Mol Sci 2014; 15:14848-14867.
8. Lin L, Ibrahim AS, Xu X, Farber JM, Avasesian V, Baquir B, et al. Th1-Th17 Cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in Mice. PLoS Pathog 2009; 5:e1000703.
9. Mencacci A, Cenci E, Del Sero G, Fe’d’Ostiani C, Mosci P, Bistoni F. Defective co-stimulation and impaired Th1 development in tumor necrosis factor/lymphotoxin-a double-deficient mice infected with Candida albicans. Int Immunol 1998; 10:37-48.
10. Ashman RB, Vijayan D, Wells CA. IL-12 and related cytokines: function and regulatory implications in Candida albicans infection. Clin Dev Immunol 2011; 2011:1-9.
11. Heizmann P, Kielisch F, Heizmann WR. Basic research – significance of detection and clinical impact of Candida albicans in non-immunosuppressed patients. PharmacoP Pharm 2011; 2:354-360.
12. Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of Candida albicans by the innate immune system. Nat Rev Microbiol 2008; 6:67-78.
13. Khosravi AR, Mardanjehmehr H, Shokri H, Naghshineh R, Rostamibashman M, Naseri A. Mycological and histopathological findings of experimental disseminated candidiasis in dogs. Iran J Vet Res 2009; 10:229-234.
14. Soltani M, Khosravi AR, Asadi F, Shokri H. Evaluation of protective efficacy of Spirulina platensis in BALB/C mice with candidiasis. J Mycol Méd 2012; 22:329-334.
15. Jimenez-Valera M, Moreno E, Amat MA, Ruiz-Bravo A. Modification of mitogen-driven lymphoproliferation by ceftriaxone in normal and immunocompromised mice. Int J Antimicrob Agents 2003; 22:607-612.
16. Brown MR, Thompson CA, Mohamed FM. Systemic candidiasis in an apparently immunocompetent dog. J Vet Diagn Invest 2005; 17:272-276.
17. Fatahnia M, Khosravi AR, Shokri H. Propolis efficacy on TNF-α, IFN-γ and IL2 cytokines production in old mice with and without systemic candidiasis. J Mycol Méd 2012; 22:237-242.
18. Elahi S, Pang G, Clancy R, Ashman RB. Cellular and cytokine correlates of mucosal protection in murine model of oral candidiasis. Infect Immun 2000; 68:5771-5777.
19. Kiatl RK, Sachanandani D, Pinney C, Lieberman MM. Cytokine production in cell culture by peripheral blood mononuclear cells from immunocompetent hosts. Clin Diagn Lab Immunol 1998; 1:78-81.
20. Mencacci A, Del Sero G, Cenci E, d’Ostiani CF, Bacci A, Montagnoli C. Endogenous interleukin 4 is required for development of protective CD41 T helper type 1 cell responses to Candida albicans. J Exp Med 1998; 187:307-317.
21. Seder RA, Le Gros G, Ben-Sasson SZ, Urban J, Finkelman FD, Paul WE. Increased frequency of interleukin 4-producing T cells as a result of polyclonal priming. Use of a single-cell assay to detect interleukin 4-producing cells. Eur J Immunol 1991; 21:1241-1247.

22. Kaposzta R, Tree P, Marodi L, Gordon S. Characteristics of invasive candidiasis in gamma interferon and interleukin-4-deficient mice: role of macrophages in host defense against Candida albicans. Infect Immun 1998; 66:1708-1717.

23. Vazquez-Torres A, Jones-Carson J, Warner WT, Balish E. Early resistance of IL-10 knockout mice to acute systemic candidiasis. Infect Immun 1999; 67:670-674.

24. Romani L, Mencacci A, Grohmann U, Mocci S, Mosci P, Pucetti P. Neutralizing antibody to interleukin 4 induces systemic protection and T helper type 1-associated immunity in murine candidiasis. J Exp Med 1992; 176:19-25.

25. Simon MM, Hochgeschwender U, Brugger U, Landolfø S. Monoclonal antibodies to interferon-γ inhibit interleukin 2-dependent induction of growth and maturation in lectin/antigen-reactive cytolytic T lymphocyte precursors. J Immunol 1996; 136:2755-2762.

26. Oykhman P, Mody CH. Direct microbicidal activity of cytotoxic T-lymphocytes. J Biomed Biotechnol 2010; 2010:1-9.

27. Norian R, Delirezh N, Azadmehr A. Evaluation of proliferation and cytokines production by mitogen-stimulated bovine peripheral blood mono nuclear cells. Vet Res Forum 2015; 6:265-271.