Effects of fungal cell wall polysaccharides and lipopolysaccharide on *in vitro* tumour necrosis factor alpha production by peripheral blood mononuclear cells of sarcoidosis patients

Učinki polisaharidov iz celične stene gliv in lipopolisaharida na *in vitro* sintezo tumorskega nekroznega dejavnika alfa v mononuklearnih celicah iz periferne krvi bolnikov s sarkoidozo

Sanja Stopinšek,[1] Marjeta Terčelj,[2] Barbara Salobir,[2] Branka Wraber,[1] Alojz Ihan,[1] Ragnar Rylander,[3] Saša Simčič[1]

[1] *Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia*

[2] *Department for Respiratory Diseases and Allergy, University Medical Centre, Ljubljana, Slovenia*

[3] *BioFact Environmental Health Research Center, Lerum, Sweden*

Korespondenca/ Correspondence:
Saša Simčič, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia,
E-mail: sasa.simcic@mf.uni-lj.si,
Tel: +386 1 543 74 91

Ključne besede:
Sarkoidoza, PBMC, TNF-α, (1→3)-β-glukan, hitin

Key words:
Sarcoidosis, PBMC, TNF-α, (1→3)-β-glucan, chitin

Citirajte kot/Cite as:
Zdrav Vestn 2010; 79: 684–9

Prispelo: 22. maj 2009,
Sprejeto: 4. jun. 2010

Izvleček

Izhodišča: Življenje v vlažnem in plesnivem okolju povezujejo z visokim dejavnikom tveganja za sarkoidozo. V zadnjem času ugotavljajo, da so v imunopatogenezo sarkoidoze morda vpletene glive. Izdelali smo model za proučevanje *in vitro* učinkov polisaharidov iz celične stene gliv na sintezo citokinov v mononuklearnih celicah iz periferne krvi (PBMC). S tem modelom smo želeli ugotoviti, ali različni polisaharidi iz celične stene gliv spodbudijo različno sintezo tumorskega nekroznega dejavnika alfa (TNF-α) v PBMC. Prav tako smo želeli preveriti, ali polisaharidi iz celične stene gliv modulirajo sintezo TNF-α, ki jo spodbudi lipopolisaharid (LPS). Nazadnje pa smo želeli ugotoviti, ali obstajajo morebitne razlike v spodbujanju sinteze TNF-α s polisaharidi iz celične stene gliv v PBMC med bolniki s sarkoidozo in zdravimi osebami.

Metode: V raziskavo je bilo vključenih enajst bolnikov s sarkoidozo in enajst zdravih oseb. Iz venske krvi bolnikov s sarkoidozo in zdravih oseb smo osamili PBMC in jih spodbujali s polisaharidi iz celične stene gliv (topnim in netopnim (1→3)-β-glukanom, cimosanom A ter hitinom) in LPS. V supernatantih celičnih kultur smo določili koncentracijo TNF-α s kvantitativno encimskoimunsko tehniko ELISA.

Razultati: Netopni (1→3)-β-glukan je bil močan spodbujevalec sinteze TNF-α v PBMC, tako pri bolnikih s sarkoidozo kot pri zdravih osebah. Topni (1→3)-β-glukan, cimosan A in hitin pa so bili šibki spodbujevalci sinteze TNF-α v PBMC tako pri bolnikih s sarkoidozo kot pri zdravih osebah. Netopni (1→3)-β-glukan in hitin sta zmanjšala, medtem ko je topni (1→3)-β-glukan povečal sintezo TNF-α, ki jo je spodbudil LPS. Pri bolnikih s sarkoidozo je bila nakazana zmanjšana sinteza TNF-α po spodbujanju PBMC s polisaharidi v primerjavi z zdravimi osebami, vendar nismo odkrili statistično značilnih razlik med bolniki s sarkoidozo in zdravimi osebami.

Zaključki: V tem članku so predstavljeni rezultati naše predhodne *in vitro* raziskave. Ugotovili smo, da različni polisaharidi iz celične stene gliv spodbudijo različno *in vitro* sintezo TNF-α v kulturah PBMC bolnikov s sarkoidozo in zdravih oseb. Prav tako smo ugotovili, da lahko nekateri polisaharidi iz celične stene gliv modulirajo sintezo TNF-α PBMC, spodbujeno z LPS, tako pri bolnikih s sarkoidozo kot pri zdravih osebah. Med bolniki s sarkoidozo in zdravimi osebami nismo dokazali statistično značilnih razlik, vendar je bila pri bolnikih s sarkoidozo nakazana manjša sinteza TNF-α po spodbujanju PBMC s polisaharidi v primerjavi z zdravimi osebami. Rezultati naše študije kažejo, da lahko izpostavljenost glivam morda vpliva na vnetni odziv v pljučih.

Abstract

Background: Living in damp and moldy environments is being associated with a high risk for sarcoidosis and recently fungi have been suspected to be involved in the immunopathogenesis of sarcoidosis. We developed a model to study the influence of fungal cell wall polysaccharides on *in vitro* cytokine production by peripheral blood mononuclear cells (PBMC). We investigated

whether different fungal cell wall polysaccharides induce different levels of tumour necrosis factor alpha (TNF-α) production by PBMC. We also assessed whether fungal cell wall polysaccharides modulate the lipopolysaccharide (LPS)-induced the TNF-α production by PBMC. And finally, we assessed whether there is a possible difference in TNF-α response of PBMC between patients with sarcoidosis and healthy persons.

Methods: Eleven patients with newly diagnosed sarcoidosis and eleven healthy volunteers were included in the study. PBMC were isolated from whole blood and stimulated with fungal cell wall polysaccharides (soluble and particulate (1→3)-β-glucan, zymosan A and chitin) and LPS. The secretion of TNF-α was measured from cultured PBMC.

Results: Particulate (1→3)-β-glucan was a potent inducer of TNF-α secretion by PBMC from patients with sarcoidosis and healthy persons. Soluble (1→3)-β-glucan, zymosan A and chitin were weak inducers of TNF-α secretion by PBMC from patients with sarcoidosis and healthy persons. Soluble (1→3)-β-glucan increased, while particulate (1→3)-β-glucan and chitin depressed the LPS induced secretion of TNF-α by PBMC from patients with sarcoidosis and healthy persons. There was no significant difference between patients with sarcoidosis and healthy persons, although there was a slight decrease of TNF-α secretion by PBMC from patients with sarcoidosis, compared to healthy persons.

Conclusions: In this article we present the results of our preliminary study. We found that different fungal cell wall polysaccharides induced different levels of TNF-α production by PBMC from patients with sarcoidosis and healthy persons. Moreover, we found that some fungal cell wall polysaccharides modulated the LPS induced TNF-α secretion by PBMC from patients with sarcoidosis and healthy persons. But we found no significant deference between patients with sarcoidosis and healthy persons, although there was a slight decrease in TNF-α secretion by PBMC from patients with sarcoidosis, compared to healthy persons. The results of our study could suggest that exposure to fungi might influence the inflammatory response of the lung.

Introduction

Sarcoidosis is a multisystem granulomatous disease which most frequently affects the lung and mediastinal lymph nodes. Its course is often benign with spontaneous regression, but in some cases it can progress to fibrosis. The diagnosis is established when clinicoradiological findings are supported by histological evidence of noncaseating granulomas. Corticosteroids are the mainstay of therapy for sarcoidosis.[1]

The immune mechanisms that cause sarcoidosis are not completely known. The process seems to begin with an antigenic stimulus, followed by T-cell and macrophage activation via a classic major histocompatibility complex (MHC) II–mediated pathway. This process has all the features of a classic T-helper 1 (Th1) response. As part of this process, cytokines and chemokines are released that recruit cells to sites of granuloma formation and trigger activation of these cells. The local environment of the granulomas eventually may change to a more T-helper 2 (Th2)-like environment that decreases the intensity of granuloma formation but favors the development of fibrosis.[2]

Despite intensive research, the etiology of sarcoidosis remains unknown. The prevailing hypothesis *is* that various antigens could promote sarcoidosis in genetically susceptible hosts.[3] Recently, there is accumulating evidence that long-term and high exposure to microbes may be involved in risk for sarcoidosis.[4] Several epidemiological studies have shown relationship between sarcoidosis and living in humid and moldy environment. Some authors suspect that fungi are involved in the immunopathogenesis of sarcoidosis.[4-7]

Fungi are ubiquitous heterotrophic organisms present in both indoor and outdoor environments. The fungal cell wall is composed of a complex of proteins and polysaccharides such as glucan, mannan, and chitin. (1→3)-β-glucan is a major fungal cell wall component, which possesses immunomodulatory activities. In inhalation studies in humans and animals, it causes a depression of macrophage function and the immune response is skewed towards a Th2-like

reactivity.[8] In *in vitro* studies it can activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activities, and stimulate the production of proinflammatory mediators.[9,10] Chitin is a polymer of N-acetylglucosamine. Recent studies demonstrated that chitin and its derivatives have effects on innate and adaptive immune responses, including the ability to recruit and activate innate immune cells and induce cytokine and chemokine production.[11]

Based on the previously described model of *in vitro* stimulation of PBMC,[12] we developed a model to study the influence of fungal cell wall components, namely (1→3)-β-glucan and chitin, on *in vitro* TNF-α production by PBMC from patients with sarcoidosis and healthy persons with reference to the effect induced by LPS and with LPS administered simultaneously. We examined whether fungal cell wall polysaccharides had a modulatory effect on the LPS-induced production of TNF-α by PBMC from patients with sarcoidosis and healthy subjects. And finally, we examined whether the TNF-α production was different between patients with sarcoidosis and healthy persons.

Methods

Subjects

Eleven patients with newly diagnosed sarcoidosis (four females and seven males; age range, 29 to 56 years) were included in the study. The patients were diagnosed at the Department of Respiratory Diseases and Allergy at the University Medical Centre Ljubljana, Slovenia, using the diagnostic criteria for sarcoidosis presented by ERS/ATS.[1] The patients had pulmonary sarcoidosis, stage II or III. No patient was receiving treatment with corticosteroids. The control group consisted of eleven healthy volunteers (seven females and four males; age range, 25 to 52 years) with no symptoms of allergy, autoimmune diseases or acute infections. The study was approved by The National Medical Ethics Committee of the Republic of Slovenia and informed consent was obtained from the participants.

Reagents

LPS from *E. coli* 0111:B4 was acquired from Sigma-Aldrich, Germany. The LPS was prepared as a 10 ng/ml dilution in the cell culture medium. As (1→3)-β-glucan we used curdlan, a linear (1→3)-β-glucan from *Alcaligenes faecalis*, from Wako Pure Chemical Industries, Ltd. (Japan). Soluble (1→3)-β-glucan was prepared by dissolving curdlan in 0.3 M NaOH (Sigma) by heating at 80°C. The pH of the soluble (1→3)-β-glucan preparation was neutralized with 0.3 M HCl (Sigma), before being added to the cell cultures. A suspension of curdlan in RPMI 1640 (Sigma) medium represented a particulate (1→3)-β-glucan. Zymosan A from *Saccharomyces cerevisiae* (Sigma) was prepared by boiling in 0.25 M NaOH, followed by centrifugation and resuspension in RPMI 1640 medium. A deacetylated derivative of chitin–chitosan, a low molecular weight chitosan from crab shells (Sigma), was dissolved in 0.2 % acetic acid. Soluble (1→3)-β-glucan, particulate (1→3)-β-glucan, zymosan A and chitin were all prepared as a 200 μg/ml concentration in cell culture medium.

Isolation and stimulation of PBMC

PBMC from patients with sarcoidosis and healthy persons were isolated by density gradient centrifugation with Ficoll-PaqueTM (Pharmacia, Sweden). The cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 2 mM L-glutamin (Sigma) and 10 % heat-inactivated AB normal human serum (Sigma). The 1 x 10^6 cells (final culture volume 1.5 ml) were plated in 24-well culture plates (Corning Costar, USA) with each of four polysaccharide compounds alone, or with a combination of a polysaccharide compound and LPS, or with medium alone, at 37 °C in a humidified atmosphere of 5 % CO_2 in the air. The cell-free supernatants were collected after 4-hour incubation and stored at -30 °C before further analysis.

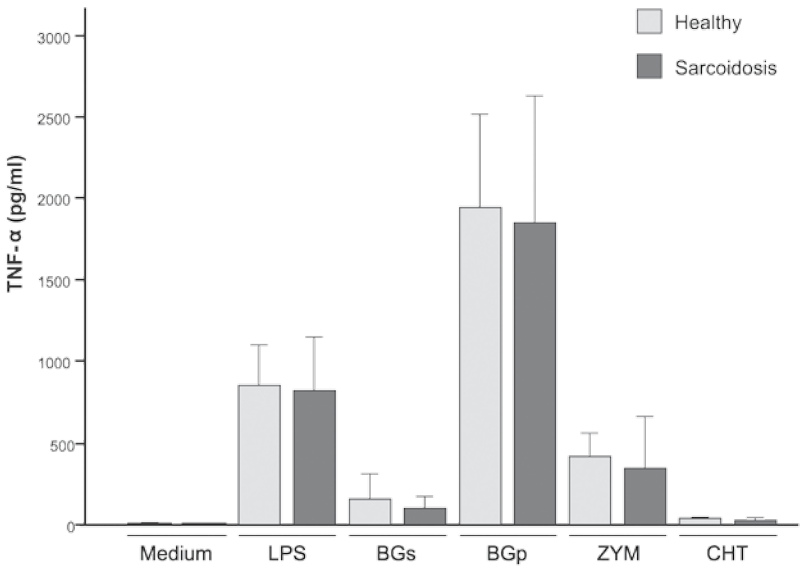

Figure 1: TNF-α production by PBMC stimulated either with LPS or with different fungal cell wall polysaccharides. BGs – soluble (1→3)-β-glucan, BGp particulate (1→3)-β-glucan, ZYM – zymosan A, CHT – chitin.

Cell viability

Cytotoxicity of polysaccharide preparations and cytolysis in cell cultures was assessed by measuring lactate dehydrogenase (LDH) activity released from damaged cells using a commercially available colorimetric assay (The Cytotoxicity Detection KitPLUS, Roche Applied Science, Germany) according to the manufacturer's instructions.

Measurement of TNF-α in cell supernatants

The concentrations of TNF-α in the cell culture supernatants were quantified using commercially available human enzyme-linked immunosorbent assay kit ELISA (Milenia Biotec, Germany) with sensitivity of 6 pg/ml.

The effect of adding LPS to the fungal polysaccharides (PS) was evaluated using an index calculated from the TNF-α value from the combined exposure and the values from each agent alone:

$$\text{Index}^{1} = \frac{\text{TNF(PS + LPS)}}{\text{TNF(PS) + TNF(LPS)}}$$

Values above 1 represent a stimulation and values below 1 a suppression. In previous studies of immunomodulatory effects, indexes below 0.8 and above 1.2 were taken as suppression and stimulation limits, because the sum of errors associated with the responses measured should be within the range index of 1 ± 0.2.[12]

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The significance of differences between means was determined using Student's t-test. For all statistical calculations, a p-value of < 0.05 was considered significant.

Results

We first assessed the ability of fungal cell wall polysaccharides to induce TNF-α production by cultured PBMC from patients with sarcoidosis and healthy persons. LPS is a well known potent proinflammatory cytokine inducer and it presented a positive control in our *in vitro* model for studying TNF-α production by PBMC. The spontaneous release of TNF-α in nonstimulated PBMC from patients with sarcoidosis and healthy subjects was under the detectable limit of 6 pg/ml. Figure 1 shows the TNF-α production induced by the different agents. Particulate (1→3)-β-glucan caused a 2-fold higher magnitude in activity than LPS. On the other hand, soluble (1→3)-β-glucan, zymosan A and chitin were weak TNF-α inducers. No significant differences were found between patients with sarcoidosis and healthy persons, although there was a slight decrease in TNF-α production by PBMC from patients with sarcoidosis compared to healthy persons.

We next examined whether fungal cell wall polysaccharides had a modulatory effect on the LPS-induced production of TNF-α by PBMC. PBMC of either patients with sarcoidosis or healthy persons were incubated with specific fungal cell wall polysaccharide in the presence of LPS. The results are shown in Figure 2. Soluble (1→3)-β-glucan showed significant stimulatory effect on the LPS-induced production of TNF-α by PBMC of patients with sarcoidosis and healthy subjects. Chitin caused suppressive effect on the LPS-induced production of TNF-α by PBMC of patients with sarcoidosis and healthy persons. Particulate (1→3)-β-glucan

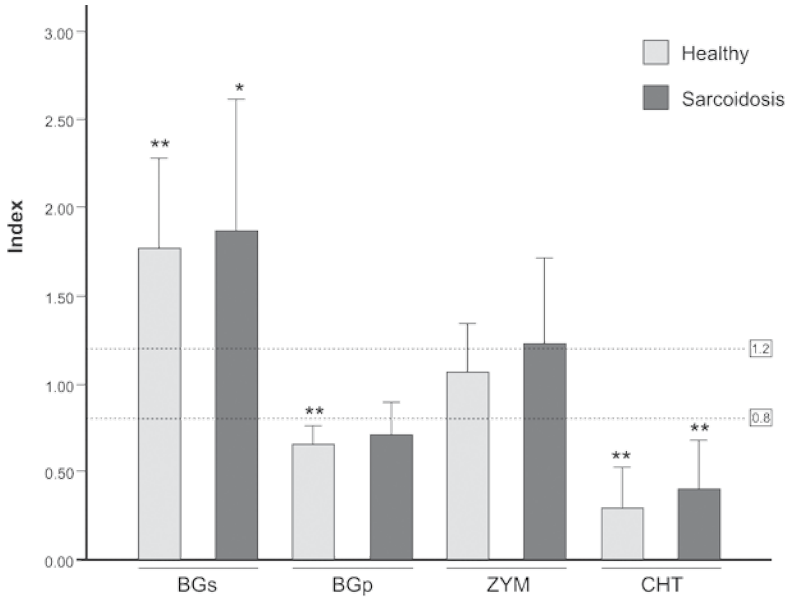

Figure 2: Modulation of LPS-induced TNF-α production by PBMC by fungal cell wall polysaccharides. BGs – soluble (1→3)-β-glucan, BGp particulate (1→3)-β-glucan, ZYM – zymosan A, CHT – chitin. *p< 0.05; **p< 0.01; Significantly different from index 1 (Student t-test).

also showed significant suppressive effect, but only in healthy persons. Zymosan A had no effect on the LPS-induced production of TNF-α by PBMC of patients with sarcoidosis and healthy persons. Modulatory effects of fungal polysaccharides on TNF-α production evoked by LPS showed no significant difference between patients with sarcoidosis and healthy persons.

Discussion

The major findings in our study were that among the different fungal polysaccharides studied, there were differences in their capacity to induce TNF-α secretion from PBMC from patients with sarcoidosis and healthy persons. There were no significant differences between healthy controls and patients with sarcoidosis. When LPS was given together with fungal cell wall polysaccharides, the TNF-α secretion was increased by soluble (1→3)-β-glucan but decreased by particulate (1→3)-β-glucan and chitin.

(1→3)-β-glucans have immunomodulatory properties at concentrations similar to those found in the environment. (1→3)-β-glucans and LPS usually occur as complexed mixtures in environmental dust.[13] Thorn et al. showed a decreased TNF-α secretion from PBMC in volunteers without indoor air problems at home after an inhalation challenge with (1→3)-β-D-glucan via a nebulizer.[14] Beijer et al. also found that an inhalation challenge with (1→3)-β-D-glucan induced a decrease in the secretion of TNF-α from LPS-stimulated PBMC.[15] These findings are supported by data from animal models, where a down-regulation of the inflammatory response after pretreatment with (1→3)-β-D-glucan has been shown. In mice given (1→3)-β-D-glucan intramuscularly, a decreased TNF-α secretion from mononuclear cells after *in vivo* stimulation with LPS was demonstrated.[16] Nakagawa et al. demonstrated that the LPS-induced IL-6 production in the PBMC cultures was significantly suppressed when soluble β-glucan from *Candida albicans* was added at the same time as the LPS and suggested that glucan suppresses the functions of activated monocytes or inhibits the activation process[17]. In our study, we investigated the *in vitro* ability of fungal cell wall polysaccharides to modulate the TNF-α production induced by LPS. Like the authors mentioned above, we found significant suppressive effect of particulate (1→3)-β-glucan. We also found that chitin had suppressive effect on the LPS-induced TNF-α production by PBMC. On the other hand, we found a significant stimulative effect of soluble (1→3)-β-glucan on LPS-induced TNF-α production by PBMC. Further research is needed to interpret these diverse effects of glucans and chitin on the LPS-induced TNF-α production by PBMC. These differences might be of importance for understanding the influence of fungal exposure for the development of sarcoidosis. Further research is needed to explore this possibility.

Conclusions

In this article, we present the preliminary results of our study of the effects of fungal cell wall polysaccharides and LPS on the *in vitro* cytokine production by PBMC. Fungal cell wall polysaccharides induced TNF-α production *in vitro* by PBMC from patients with sarcoidosis and healthy persons. In continuation of our study, we intend to discover possible differences between sarcoidosis patients and healthy persons in cytokine production *in vitro* by PBMC after stimulation with fungal cell wall polysaccharides.

The results of our study could confirm an involvement of fungi in the immunopathogenesis of sarcoidosis.

References:

1. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. Am J Respir Crit Care Med 1999; 160: 736–5.
2. Gerke AK, Hunninghake G. The Immunology of Sarcoidosis. Clin Chest Med 2008; 29: 379–390.
3. Nunes H, Soler P, Valeyre D. Pulmonary sarcoidosis. Allergy 2005; 60: 565–82.
4. Newman LS, Rose CS, Bresnitz EA, Rossman MD, Barnard J, Frederick M, et la. A case control etiologic study of sarcoidosis: environmental and occupational risk factors. Am J Respir Crit Care Med 2004; 170: 1324–30.
5. Tercelj M, Rott T, Rylander R. Antifungal treatment in sarcoidosis-a pilot intervention trial. Resp med 2007; 101: 774–8.
6. Tercelj M, Salobir B, Rylander R. Microbial antigen treatment in sarcoidosis-a new paradigm? Medical hypotheses 2008, 70: 831–4.
7. Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. N Engl J Med 2007; 357: 2153–65.
8. Rylander R. (1-3)-β-D-glucan in the environment a risk assessment. V: Young SH and Castranova V., ur. Toxicolocy of 1-3-β-D-glucan. Boca Raton: Taylor and Francis; 2005. Str. 139-154.
9. Brown GD, Gordon S. Fungal beta-glucans and mammalian immunity. Immunity 2003; 19: 311–5.
10. Young SH, Ostroff GR, Zeidler-Erdely PC, Roberts JR, Antonini JM, Castranova V. A comparison of the pulmonary inflammatory potential of different components of yeast cell wall. J Toxicol Environ Health 2007; 70: 1116–24.
11. Lee CG, Da Silva CA, Lee JY, Hartl D, Elias JA. Chitin regulation of immune responses: an old molecule with new roles. Curr Opin Immunol 2008; 20: 684–9.
12. Simcic S, Wraber B, Sollner M, Urleb U, Gobec S. Modulation of tumor necrosis factor production with desmuramyldipeptide analogues. Eur J Physiol Curr Opin Immunol 2000; 440 Suppl 5: R64-R66.
13. Rylander R, Holt PG. (1-> 3)-β-D-Glucan and endotoxin modulate immune response to inhaled allergen. Mediators Inflamm 1998; 7: 105–10.
14. Thorn J, Beijer L, Rylander R. Effects after inhalation of (1—> 3)-beta-D-glucan in healthy humans. Mediators Inflamm 2001; 10: 173-8.
15. Beijer L, Thorn J, Rylander R. Effects after inhalation of (1-> 3)-β-D-Glucan and relation to mould exposure in the home. Mediators Inflamm 2002; 11: 149–53.
16. Soltys J, Quinn MT. Modulation of endotoxin- and enterotoxin-induced cytokine release by in vivo treatment with beta-(1,6)-branched beta-(1,3)-glucan. Infect Immun 1999; 67: 244-52.
17. Nakagawa Y, Ohno N, Murai T. Supression by *Candida albicans* β-Glucan of cytokine release from activated human monocytes and from T cells in the presence of monocytes. J Infect Dis 2003; 187: 710–3.