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IL-6 mRNA Expression in Mouse Peritoneal Macrophages and NIH3T3 Fibroblasts in Response to *Candida albicans*

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Abstract Despite extensive investigation, the mechanisms of immune responses to *Candida albicans* infection remain poorly understood. Using RT-PCR and Northern blot analysis, this study demonstrates the pattern of IL-6 mRNA expression in thioglycollate-elicited mouse peritoneal macrophages and NIH3T3 fibroblasts (NIH3T3) in response to *C. albicans*. The expression of IL-6 mRNA was detectable in both cell types. However, IL-10 mRNA was only expressed in the macrophages, and IL-4 mRNA was not expressed in neither of the two cell types. Although the phagocytic function of the macrophages was inhibited by Cytochalasin D, these macrophages could still induce the expression of IL-6 mRNA. These findings indicate that the phagocytosis of *C. albicans* is not pivotal in the induction of IL-6 mRNA expression. A Northern blot analysis was used to investigate the dose effects of *C. albicans* and time-course kinetics of IL-6 mRNA expression at various time points. IL-6 mRNA was expressed in a dose-independent manner, and was detectable as early as 30 min after *C. albicans* stimulation. It was evenly sustained up to 4 h. These results can contribute to understanding the mechanism of IL-6 mRNA expression in macrophages and NIH3T3 cells in response to *C. albicans*.

Key words: IL-6, mouse peritoneal macrophages, NIH3T3 fibroblasts, *Candida albicans*

C. albicans is a ubiquitous yeast found on many plants. It is also part of the normal flora of the alimentary tract and mucocutaneous membranes of humans, and is one of the common fungal pathogens in humans and animals that cause opportunistic infections, such as candidiasis [1, 3, 18, 20]. Although it has been postulated that a defect in cell mediated immunity is critical for this disease to occur, the exact mechanism of candidiasis is not yet clear.

It is widely acknowledged that cytokines play an important role in the regulation of inflammatory responses as well as in the generation of immunity to pathogens [30]. Interaction with microbes causes the secretion of a variety of cytokines by many cells including neutrophils, macrophages, endothelial cells, and fibroblasts. However, details regarding the induction of Th2-type cytokines (IL-4 mRNA, IL-6 mRNA, IL-10 mRNA) by microbes are still unclear, even though extensive studies regarding cytokine induction by microbial antigens and infections have been previously reported [11, 12, 23, 27, 31].

Macrophages are known as major effector cell types, and synthesize a variety of immunomodulatory factors, including cytokines, and leukocyte adhesions [2, 13, 19]. Cytokine synthesis by macrophages is likely to occur during the interaction of macrophages with microbes during phagocytosis. Mesenchymal cells such as fibroblasts have also been shown to be important effector cells in inflammatory reactions, including the regulation of wound healing and remolding of connective tissue [4, 21]. Therefore, it is probable that these cells play a significant role in determining the magnitude and profile of the host's inflammatory response to *C. albicans*.

The purpose of the present investigation was to gain information on the expression of IL-4, IL-6, and IL-10 mRNA in thioglycollate-elicited mouse peritoneal macrophages and NIH3T3 fibroblasts cells in response to *C. albicans*. It is acknowleged that phagocytosis plays an important role in some cytokine expression [31]. Therefore, the second objective of this study was to gain insights into the relationship between IL-6 mRNA expression and phagocytosis of *C. albicans* in thioglycollate-elicited mouse peritoneal macrophages.

MATERIALS AND METHODS

Media and Chemicals

RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS), L-glutamine,

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trypsin, and Fetal Bovine Serum (FBS) were purchased from Gibco BRL (Grand Island, U.S.A.). FBS was inactivated at 56°C for 30 min. Primers (IL-4, IL-6, IL-10) for the RT-PCR were synthesized from Bioneer Corp., (Chungbuk, Korea). Lipopolysaccharide (LPS: *E. coli* 0111: B4) and Cytochalasin D were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). RNAzol B for the total RNA isolation was purchased from Biotex Laboratories INC., U.S.A. The RT-PCR kits and High Prime were obtained from Boehringer Mannheim GmbH (Germany). IL-6 plasmid was generously provided by Dr. Hamilton (Research Institute, Cleveland Clinic Foundation, U.S.A.). [α-^{32}P]-dCTP was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). All other reagents were commercial preparations of pure grade.

Microorganism

C. albicans B311 was kindly provided by Dr. D. Y. Ha at the College of Medicine, Chunbuk National University. The organisms were grown on Sabouraud's dextrose agar (SDA:Difco Laboratories, Detroit, U.S.A.) for 76 h at 37°C and harvested with phosphate buffered saline (PBS). The number of yeast cells in the RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% FBS was adjusted to 1×10^7/well in 6-well plates for the RT-PCR. The yeast form was examined by microscopy to be present throughout the experiments. In the case of the cytochalasin D (CyD) experiment, CyD was added to the medium at a final concentration of 0.2 μM immediately before the addition of *C. albicans*, and the control macrophages were exposed to an equal amount of the diluents (dimethyl sulfoxide; final concentration, 0.02%) prior to innoculation.

Macrophages

Specific pathogen-free, 9-12 weeks old female inbred BALB/c mice were purchased from Hyeuchang Sci. Corp., (Taegu, Korea). They were provided with autoclaved food, water, and fed and maintained in minimal-stress conditions so that the mice remained free from infection by environmental pathogens to ensure that the degree of spontaneous activation of tissue macrophages would be minimal. Thioglycollate-elicited macrophages were obtained using Hamilton's method [13]. Peritoneal larvage was performed using 10 ml of cold HBSS containing 10 U/ml of heparin. The macrophages in the culture medium were plated in 6-well plates, incubated for 2 h at 37°C in an atmosphere of 5% CO_2, washed three times with HBSS to remove any nonadherent cells, and then equilibrated in the culture medium for 18 h prior to the initiation of each experiment. In most experiments, the macrophages were cultured overnight, and then washed once with the complete medium. After washing, stimuli were added at the following concentrations. *C. albicans* was added at a ratio of 10 yeast cells per macrophage and treated with *E. coli* lipopolysaccharide (LPS: O111:B4) at a final concentration of 100 ng/ml. The control cells were not stimulated with LPS or any other stimulator.

NIH3T3 Fibroblasts

NIH3T3 fibroblasts were generously provided by Dr. Hamilton at the Research Institute, Cleveland Clinic. Foundation, U.S.A. NIH3T3 fibroblasts were cultured in DMEM supplemented with 10% FBS containing L-glutamine, penicillin, and streptomycin, and subcultured twice weekly. Before use in the experiments, the concentration of cells was adjusted to 1×10^6/well in a 6-well plate. NIH3T3 monolayers were cultured overnight and then washed once with the complete medium. After washing, stimuli were added at the following concentrations. *C. albicans* was added at a ratio of 10 yeast cells per 1 NIH3T3 cell and treated with *E. coli* lipopolysaccharide (LPS; O111:B4) at a final concentration of 100 ng/ml. The control cells were not stimulated with LPS or any other stimulator.

Total RNA Isolation and RT-PCR

Total RNA from the macrophges and NIH3T3 cells was extracted using RNAzolB according to the manufacturer's instructions (Biotex Laboratories INC., U.S.A.). An RT of

Table 1. Oligonucleotide primers and predicted PCR product sizes for cytokine cDNAs.

mRNA	Sence primer	Antisence primer	Predicted size of PCR product (bp)
IL-4	5'-ACA AAA ATC ACT TGA GAG AGA TCA T-3'	5'-AGT AAT CCA TTT GCA TGA TGC TCT T-3'	351
IL-6	5'-CTG GTG ACA ACC ACG GCC TTC CCT A-3'	5'-ATG CTT AGG CAT AAC GCA CTA GGT T-3'	600
IL-10	5'-ACC TGG TAG AAG TGA TGC CCC AGG CA-3'	5'-CTA TGC AGT TGA TGA AGA TGT CAA A-3'	237
β2-Microglobulin	5'-GGC TCG CTC GGT GAC CCT AGT CTT T-3'	5'-TCT GCA GGC GTA TGT ATC AGT CTC A-3'	300

*The primer sequences have been discussed in detail in recent reviews [9]

the total RNA was performed with avian myeloblastosis virus reverse transcriptase in a commercial reaction mixture. The primers for the PCR are listed in Table 1. A PCR amplification was performed in a Perkin Elmer (The Perkin-Elmer Corp., Norwalk, U.S.A.) for 35 cycles as described previously [17]. The first cycle consisting of a 5 min denaturation at 94°C and a 5 min annealing at 60°C was followed by 35 cycles of 1.5 min at 72°C, 45s at 94°C, and of 45s at 60°C, with a final extension of 10 min at 72°C. The PCR products were analyzed by electrophoresis with an 8% polyacrylamide gel and visualized by 0.5 µg/ml ethidium bromide staining. The reported data were representative of at least three different experiments. Under these conditions, control samples taken from uninfected macrophages showed no background cytokine mRNA levels; accordingly, the magnitude of the immune response to infection could be easily demonstrated.

Transmission Electron Microscopy Preparation

Macrophages were grown for 24 h and *C. albicans* was added at a ratio of 10 yeast cells per 1 macrophage. In the cytochalasin D (CyD) experiment, CyD was added to the medium at a final concentration of 0.2 µM immediately before the addition of *C. albicans*, and the control macrophages were exposed to an equal amount of the diluents (dimethyl sulfoxide; final concentration, 0.02%) prior to inoculation. A transmission electron microscopic analysis of *C. albicans*-infected macrophages treated with and without CyD was performed to evaluate the phagocytosis morphologically. After a 2 h incubation, the supernatant was removed and prefixated with 2.5% glutaraldehyde for 4 h and washed with a 0.1 M phosphate buffer. For postfixations, the cells were treated with 1% OsO_4 for 1 h 30 min and washed with a 0.1 M phosphate buffer. The cells were embedded in epoxy resin, which was polymerized for 48 h at 60°C. Ultrathin sections were poststained with 2% uranyl acetate and examined.

Northern Blotting

Northern blotting was performed by a standard technique [24]. Briefly, 10 µg of the total RNA was electrophoresed through 1% agarose/2.2 M formaldehyde gels and then transferred to a nylone membrane using the capillary transfer method. The membranes were hybridized with an IL-6 cDNA probe labeled with [α-^{32}P]-dCTP by the high primer method. The gene expression was detected using autoradiography and then quantified by a computer software program (Vibler Lourmat Bio 1 V 6.23). The results were normalized to the expression of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to correct for any differences in the amount of RNA added to each lane. The sample value was calculated from the simple formula: Standard GAPDH value=Standard GAPDH density value/Sample GAPDH density value, Sample value=(Standard GAPDH value × Sample density value)/Standard sample density value.

Results

IL-4, IL-6, and IL-10 mRNA Expressions in Macrophages and NIH3T3 Fibroblasts by *C. albicans* Stimulation

To examine the effect of *C. albicans* on the expression of IL-4, IL-6, and IL-10 mRNAs in macrophages and NIH3T3 fibroblasts, the cells were treated with *C. albicans* for 2 h at a 1:10 (macrophages or NIH3T3: yeasts) ratio. The expression of IL-6 mRNA was observed in both cell types. IL-10 mRNA was detectable in only the macrophages and IL-4 mRNA expression was not observed in either of the cell types (Fig. 1).

IL-6 mRNA Expression of Macrophages by *C. albicans*

To investigate whether the phagocytosis of *C. albicans* was necessary for IL-6 mRNA expression in macrophages, Cytochalasin D was used. Cytochalasin D has been used extensively for the inhibition of phagocytosis of many bacteria [11, 25, 26], and has been found to have little effect on the attachment of bacteria. The experimental conditions were similar to those previously reported [11]. Clear differences in the extent of phagocytosis between the CyD-treated and nontreated macrophages were observed. The untreated macrophages showed phagocytosed *C. albicans* within the phagosomes. An RT-PCR analysis of *C. albicans*-stimulated macrophages treated with and without CyD was performed to investigate IL-6 mRNA expression. There were no differences in the expression of IL-6 mRNA in the stimulated macrophages between the CyD-treated and untreated cells. To determine the effects of DMSO (CyD diluent) on cytokine gene expression, the control macrophages were exposed to an equal amount of diluents (DMSO) prior to inoculation. It was observed that the DMSO had no effect on cytokine gene expression. These results suggest that the attachment of *C. albicans* to the macrophage surface is sufficient to induce IL-6 mRNA expression (Fig. 2).

Effect of *C. albicans* Dose on IL-6 mRNA Expression in Macrophages

To investigate whether a differential concentration of *C. albicans* would result in different patterns of cytokine expression, various amounts (1:1, 1:10, 1:100; macrophages or fibroblasts to yeasts) of *C. albicans* were used to stimulate the macrophages and NIH3T3 cells. All three concentrations were able to induce IL-6 mRNA expression in macrophages, however, the levels of induced IL-6 mRNA were not dependent on the dose of *C. albicans*

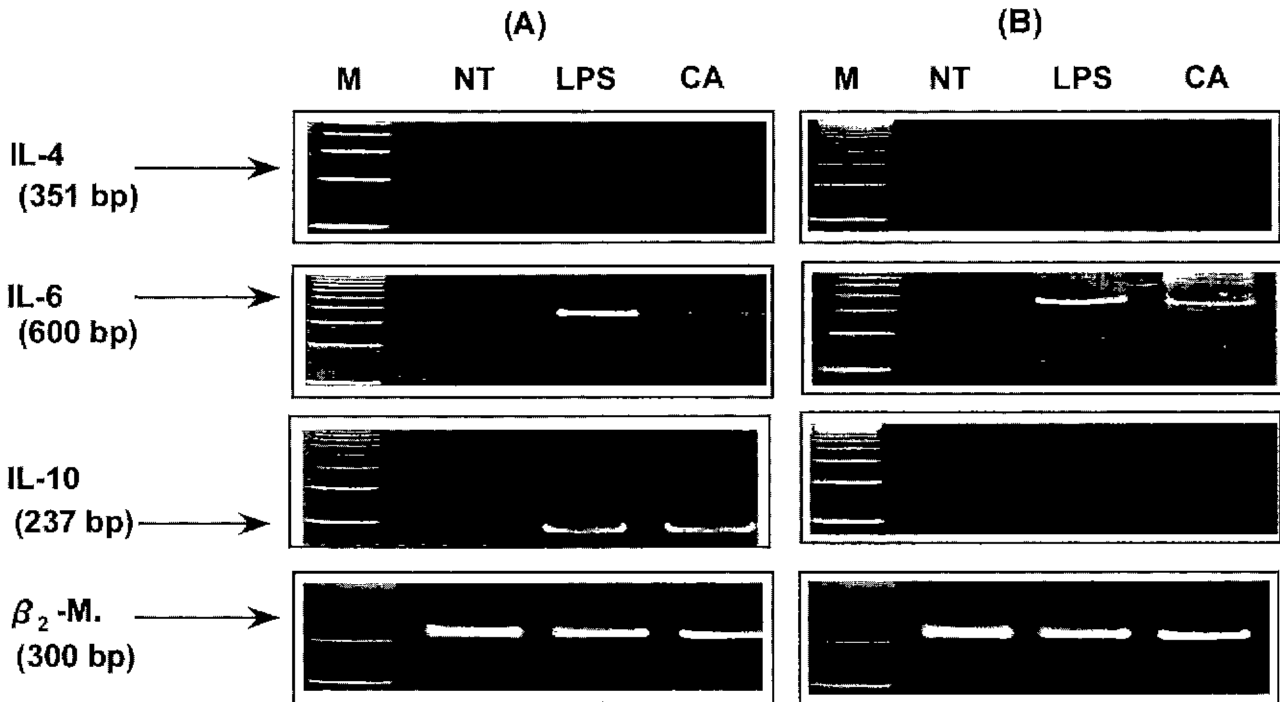

Fig. 1. *C. albicans* selectively induces Th2-type cytokines (IL-4, Il-6, IL-10 mRNA) expression.
A: Thioglycollate-elicited macrophges were stimulated with *C. albicans* at a ratio of 1:10 (target cells:yeasts) and LPS (100 ng/ml) for 2 h. B: NIH3T3 fibroblasts were stimulated with *C. albicans* at a ratio of 1:10 (target cells:yeasts) and LPS (100 ng/ml) for 2 h. Total RNA from both cell types was extracted using RNAzol B and RT-PCR was performed. LPS: Lipopolysaccharide; CA. *C. albicans*, β_2-M: β_2-microglobulin; M. 100 bp size marker. NT: Not-treated

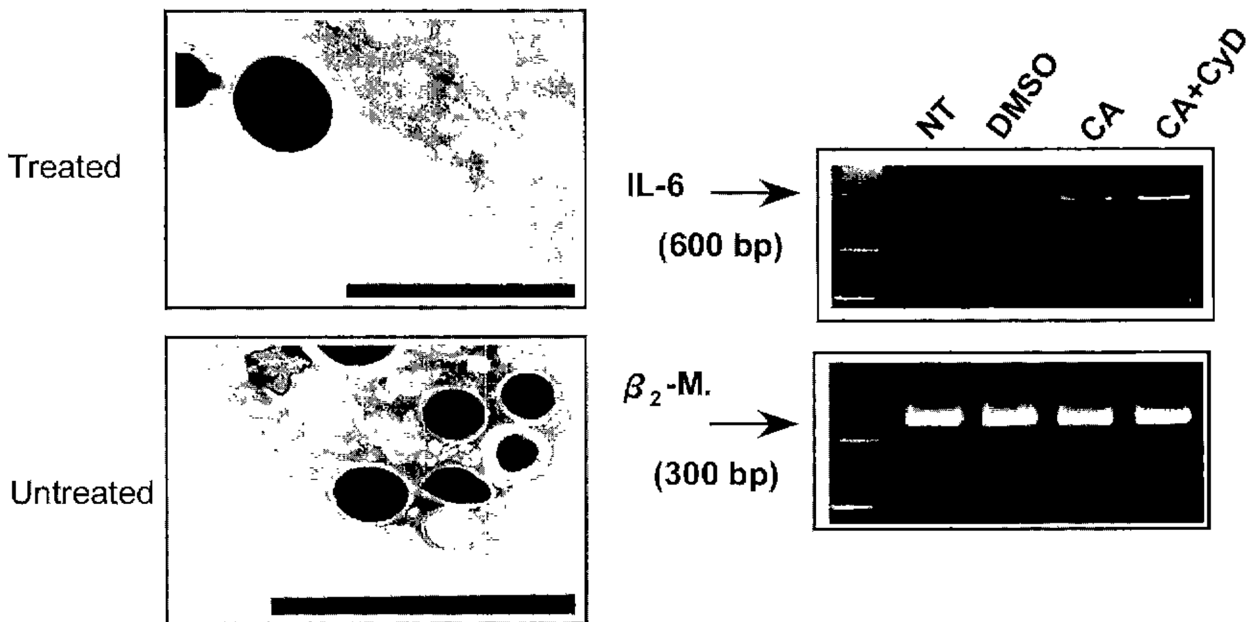

Fig. 2. The effect of phagocytosis on cytokine mRNA expression in thioglycollate-elicited mouse peritoneal macrophages induced by *C. albicans*.
Left: Electron microscopic analysis of phagocytosis in Cytochalasin D (CyD) treated macrophages. Macrophages were treated with and without 0.2 μM of CyD and simultaneously infected with *C. albicans*. Treated: No phagocytosed *C. albicans* (◉) was observed in the presence of CyD. However, the attachment to macrophages by *C. albicans* (◉) was observed. Untreated: In the absence of CyD, many *C. albicans* cells were observed in the macrophages. Bar, 4 μm. Right: Thioglycollate-elicited macrophages were treated with CyD (0.2 μM in a final concentration) and simultaneously with *C. albicans* for 2 h. After stimulation, total RNA was isolated from the macrophages, and an RT-PCR was performed with the primers indicated, as described in Materials and Methods. β_2-M: β_2-microglobulin; CA. *C. albicans*; CyD: Cytochalasin D; DMSO: dimethyl sulfoxide (0.02%, as a control medium).

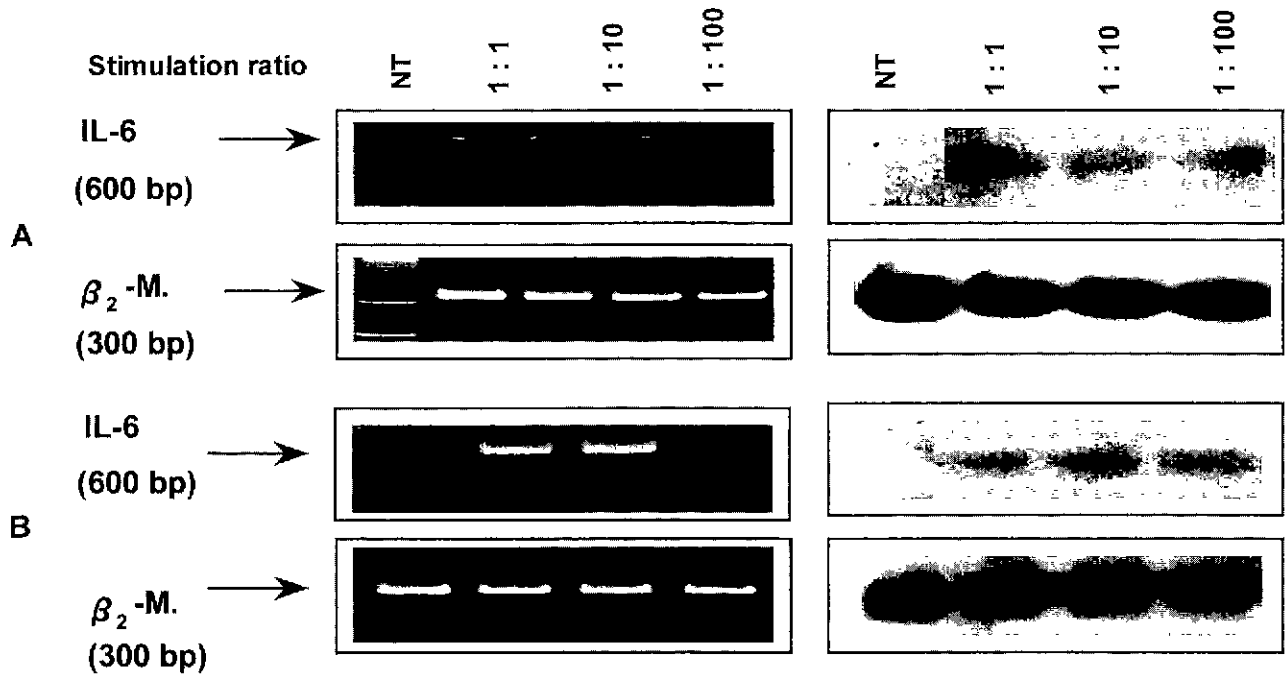

Fig. 3. Dose effect of *C. albicans* on cytokine mRNA expression in mouse peritoneal macrophages and NIH3T3 fibroblasts.
A: NIH3T3 fibroblasts were stimulated with various amounts (1:1, 1:10, 1:100) of *C. albicans* for 2 h. B: Thioglycollate-elicited macrophges were stimulated with various amounts (1:1, 1:10, 1:100) of *C. albicans* for 2 h. Total RNA was prepared from each sample and an RT-PCR (Left) and Northern blot (Right) were performed as described in Materials and Methods. M:100 bp size marker; NT: Not-treated; β_2-M β_2-microglobulin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase housekeeping gene) were used as control genes

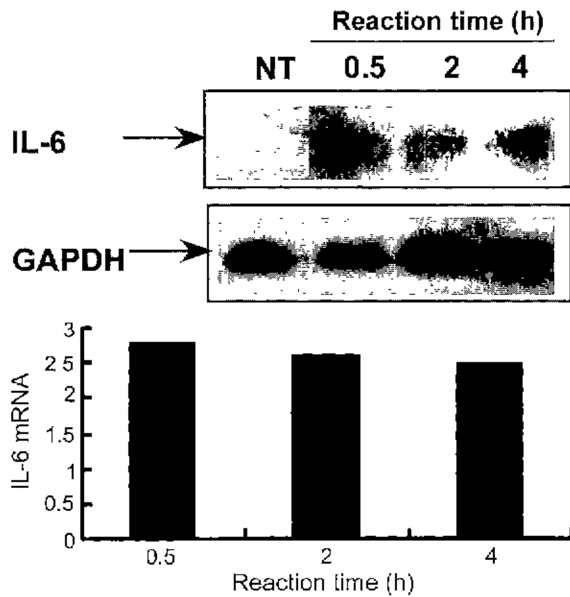

Fig. 4. Time course analysis of IL-6 mRNA expression in mouse peritoneal macrophages elicited by *C albicans*.
Thioglycollate-elicited macrophages (2×10^6/well) were cultured for various periods of time in the presence of *C albicans* (2×10^7/well) After stimulation, total cellular RNA was isolated from the cultured cells using RNAzolB and a Northern blot was performed to measure the IL-6 mRNA and GAPDH mRNA levels. Upper: Northern blot of IL-6 mRNA Lower: Time course analysis sample value of IL-6 mRNA levels GAPDH (glyceraldehyde-3-phosphate dehydrogenase housekeeping gene) was used as the control gene.

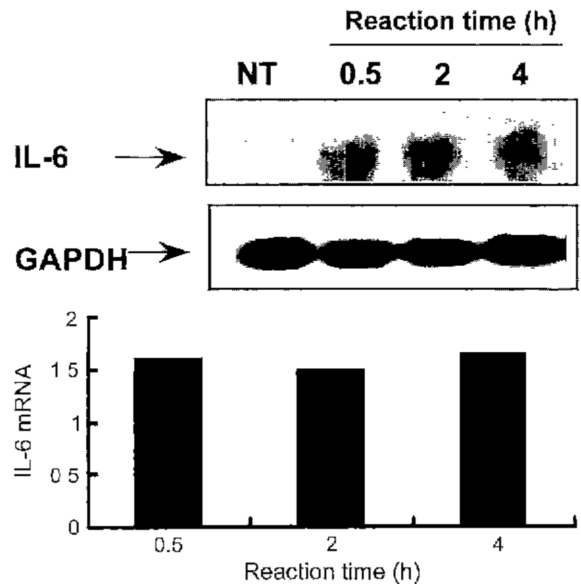

Fig. 5. Time course analysis of IL-6 mRNA expression in NIH3T3 fibroblasts elicited by *C. albicans*.
NIH3T3 fibroblasts (2×10^6/well) were cultured for various periods of time in the presence of *C. albicans* (2×10^7/well). After stimulation, total cellular RNA was isolated from the cultured cells using RNAzolB and a Northern blot was performed to measure the IL-6 mRNA and GAPDH mRNA levels. Upper: Northern blot of IL-6 mRNA; Lower: Time course analysis sample value of IL-6 mRNA levels. GAPDH (glyceraldehyde-3-phosphate dehydrogenase, housekeeping gene) was used as the control gene

used. At the 1:100 ratio, IL-6 mRNA expression was lower than at the 1:1 and 1:10 ratios. There were no differences in the expression of cytokine mRNAs between a ratio of 1:1 and 1:10 (Fig. 3). NIH3T3 cells stimulated with various amounts of *C. albicans* expressed IL-6 mRNA equally at any ratio.

Time Kinetics on IL-6 mRNA Expression in Macrophages and NIH3T3

To examine the time course of *C. albicans*-induced IL-6 mRNA expression, various time periods (0.5, 2, and 4 h) were used. A Northern blot analysis was used to quantitatively measure the IL-6 mRNA at various times after *C. albicans* stimulation. The results were normalized to the expression of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to correct for any differences in the amount of RNA added to each lane. IL-6 mRNA expression was detected in both cell types as early as 30 min, and evenly sustained for up to 4 h after *C. albicans* stimulation (Figs. 4 and 5).

Discussion

Host resistance against infection caused by *C. albicans* is mediated predominantly by neutrophils and macrophages [30]. In systemic candidiasis in mice, cytokine production has been found to be a function of the CD4+ T helper (Th) cells. The Th1 subset of these cells, characterized by the production of gamma interferon and IL-2, is associated with macrophage activation and enhanced resistance against reinfection, whereas the Th2 subset which produces IL-4, IL-6, and IL-10 mRNA is linked to the development of chronic disease [17]. Current data suggest that Th2-type cytokine response is associated with susceptibility to mucosal candidiasis [16]. However, the mechanisms of Th2 type cytokine expression in various cell types by *C. albicans* infection are not clear.

This study examined IL-4, IL-6, and IL-10 mRNA gene expression in thioglycollate-elicited murine peritoneal macrophages and NIH3T3 fibroblast cells. The expression of IL-6 mRNA was detectable in both cell types. The cytokine gene expression of *C. albicans*-induced macrophages showed the same patterns as the LPS-induced one. Namely, *C. albicans*-induced or LPS-induced cytokine gene expressions were selective to macrophages and NIH3T3, thus providing functional clues as to what comprises an efficient state of the immune response in normal human subjects.

IL-4 mRNA has pleiotropic effects on a variety of immune and nonimmune cells, because it induces the expression of a MHC class II antigen on B cells and monocytes, and enhances macrophage tumoricidal activity [7, 26]. In this experiment, *C. albicans* did not induce IL-4 mRNA expression in either of the two cell types. One possible explanation for the lack of IL-4 mRNA could be due to the extremely short half-life of this molecule. Accordingly, a specific message for this cytokine could not be detected.

IL-10 mRNA has been implicated in the regulation of the function of lympoid and myeloid cells, because of their ability to suppress the synthesis of proinflammatory cytokine from T cells and monocytes/macrophages [5]. In this experiment, IL-10 mRNA expression on macrophages induced by *C. albicans* was not detected in the fibroblasts. The difference in response between these cell types may be attributable to the cell-type specific manner of IL-10 mRNA expression. However, confirmation of these results will require further study.

IL-6 mRNA is known as an inducer of the metalloproteinase inhibitor in fibroblasts, provides a second signal for T cell activation, and promotes the growth and differentiation of B cells [6]. This suggests a potential role for IL-6 in the fibrotic response during chronic inflammation and in local immune response [21]. Since *C. albicans* induced IL-6 mRNA expression in both cell types, the experiments were focused on IL-6 mRNA expression in both cell types after exposure to *C. albicans*.

Phagocytosis in macrophages is an important host defense mechanism, and is a pivotal in the induction of certain cytokines. Yaol *et al.* [31] suggested that the phagocytosis of intact *S. aureus* by endothelial cells is important for the induction of IL-6 mRNA accumulation in endothelial cells. However, Yamamoto *et al.* [30] reported that the attachment of *C. albicans* to macrophages was sufficient to generate a signal for the induction of increased steady-state levels of certain cytokines. Yamamoto's data are in good agreement with the results of this study. Wang *et al.* [28] suggested that *C. albicans* manoprotein is important for the induction of cytokines. Therefore, the interaction of certain microbial surface ligands and their corresponding receptor on macrophages appears to be important for the induction of mRNA synthesis in selected cytokines.

There have been several studies on the effect of *C. albicans* concentrations on cytokine gene expression. Djeu *at el.* [8] reported that TNFα expression was the highest at a ratio of 1:10 (peripheral blood mononuclear cell:*C. albicans*), and Kim *at el.* [14] reported that the highest level of TNFα production was induced at a ratio of 1:50 (human peripheral blood mononuclear cell:*C. albicans*). Yamamoto *et al.* [29] reported that IL-6 mRNA expression was highest at a ratio of 1:100 (macrophages:*L. pneumophila*). In the present study on the expression of IL-6 mRNAs in macrophages, all three concentrations were able to induce IL-6 mRNA. These data support the notion that cytokine expression is involved in the differential response to the concentration of microbe and effector cells.

Kim *et al.* [6, 15] reported that the expression of IL-6 mRNA in human peripheral lymphocytes is stimulated with heat-killed *C. albicans* and the expression was detected as early as 30 min and lasted for 48 h after stimulation. Matthes *et al.* [19] reported that various mitogens-stimulated human B cells induced cytokine genes immediately after stimulation and appeared for up to 120 h. Fan *et al.* [10] reported that the parahemagglutinin (PHA) induced IL-6 mRNA expression occurred between 1 and 4 h after stimulation. In this study, IL-6 mRNA expression of *C. albicans*-induced macrophages was detected as early as 30 min and up to 4 h after stimulation. This wide range of results from different laboratories is probably due to the difference in the cell types, stimulants, and experimental environments.

In conclusion, our observations suggest that *C. albicans* can induce IL-6 mRNA expression in both thioglycollate-elicited murine peritoneal macrophages and NIH3T3 fibroblasts cells, the macrophage phagocytosis of *C. albicans* is not pivotal in the induction of IL-6 mRNA, and IL-6 mRNA expression is dose independent. Taken together, these results may contribute to the understanding of the mechanism underlying the IL-6 mRNA expression and the co-host relationship after *C. albicans* infection.

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