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BLyS and APRIL expression in peripheral blood mononuclear cells of cryptococcal meningitis patients and their clinical significance

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ABSTRACT

Objective: To investigate the levels of APRIL, BlyS and receptors as TACI, BCMA and BAFF-R in peripheral blood mononuclear cells (PBMC) of cryptococcal meningitis (CM) patients and its clinical significance.

Methods: PBMC from 30 CM patients and 32 healthy controls were isolated. The mRNA levels of APRIL, BLyS and BLyS receptors were detected by fluorescent quantitation PCR. The effect of PBMC from CM patients on *in vitro* growth of *Cryptococcus neoformans* was compared in presence and absence of BLyS.

Results: PBMC of CM patients exhibited significantly lower BLyS, TACI and BCMA mRNA levels but significantly higher BAFF-R mRNA levels than controls. Growth of *C. neoformans* was significantly slower in presence of BLyS than its absence.

Conclusion: Levels of BlyS and its receptors correlated with cryptococcal meningitis progression, and provide new clues for monitoring CM conditions and its effective therapy.

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Introduction

Cryptococcus sp. are present in the environment, especially pigeon guano and eucalyptus trees. *Cryptococcus neoformans* is an opportunistic pathogen and causes the disease called cryptococcosis [1]. Although *C. neoformans* infection can disseminate into most organs and induce a range of diseases from arthritis to sinusitis, the most frequent disease in the clinic is cryptococcal meningitis. Clinically, anti-fungal pharmaceutical drugs are widely employed to treat cryptococcal meningitis [2]. Due to rising antifungal drug resistance and the adverse side effects of antifungal therapy, there is an urgent need for elucidation of disease mechanisms of cryptococcal meningitis that can spur novel treatments. A robust immune system including an innate, cellular and humoral immune response can usually prevent cryptococcosis and provides a more rapid resolution of *C. neoformans* infection. Cryptococcal meningitis occurs predominantly in immunocompromised subjects with AIDS or organ transplantation [3,4], and it significantly raises their morbidity [5,6]. Patients with an impaired T cell immunity caused by AIDS or organ transplantation can often be protected from cryptococcosis by a high titer of anti-*Cryptococcus* antibodies produced by their B cells [7,8]. In China, cryptococcal meningitis mainly attacks non-AIDS afflicted people [9]. Understanding the role of B cells in protection from cryptococcal infections will shed light on the surveillance mechanisms for *C. neoformans* and on potential effective treatments of this disease [10,11].

Efficient B cell development and maturation require the interaction of monocytes, helper T cells as well as several critical cytokines. The B lymphocyte stimulating factor family belongs to the TNF family of growth activators and contains 2 members: B lymphocyte stimulating factor (BLyS) and A PRoliferation Inducing Ligand (APRIL). BLyS is also known as B cell activating factor (BAFF) [12]. Human BLyS gene is located in chromosome 13q32–34, and its mRNA is 2.6 kbp in length [13] whereas APRIL is encoded on chromosome 17 [14]. BLyS binds to three receptors: transmembrane activator and calcium modulator cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA) and BAFF receptor (BAFF-R). BCMA and TACI are expressed on naïve and primary B cells of follicular and germinal centers whereas B cell maturation antigen (BCMA) is found on long lived, marrow-derived, plasma B cells [15]. Constitutive BLyS produced by stromal cells governs the size of the B cell pool whereas inducible BLyS secreted by monocytes, neutrophils, dendritic cells and macrophages influences local survival of B cells [12,16]. BLyS also boosts TH1 activation, and the recombination needed for class switching of immunoglobulins important for maturation of a primary antibody response to an IgG response by interacting with the 3 receptors [17]. APRIL can specifically bind to TACI and BCMA and

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influences survival of mature plasma B cells [14,18]. An additional receptor for APRIL, APRIL-R, has yet to be identified. Thus, BlyS and APRIL play important roles in development and maintenance of humoral immunity [19–21].

Aberrant expression of BlyS and APRIL correlates with several human diseases. Elevated expression of BLyS/BAFF has been documented in several autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [22,23]. High APRIL levels are found in several B cell malignancies [18]. Conversely, mutations of the TACI receptor are causal for an immunodeficiency disease [24]. These data suggest that the BLyS family plays key roles in humoral immunity.

Prevention and resolution of cryptococcal infections correlate with an intact cellular and humoral immunity. The present study utilizes RFQ-PCR to analyze mRNA expression of BlyS, APRIL and BLyS receptors in peripheral blood mononuclear cells of cryptococcal meningitis patients and analyzes its correlation to the disease.

Materials and methods

Subjects

Thirty subjects who were diagnosed with cryptococcal meningitis in Changzheng Hospital, Shanghai by Gram's stain and/or positive culture for *C. neoformans* in their cerebral spinal fluid were enrolled in this study from August 2004 to April 2008. The patients (10 male, 20 female) had an average age of 37 years (31–62 years) and were compared to 32 healthy control subjects (12 male, 20 female) from the same hospital clinic. The healthy controls did not display fever, headache, vomiting or sore throat, and their blood biochemistry including liver functionality, kidney functionality and lipid profile was within normal standards. All subjects were HIV negative. The study obtained informed consent from the patients and controls, and approval from the Ethics review committee of Changzheng Hospital, Shanghai.

Methods

Isolation and purification of PBMC

Peripheral blood mononuclear cells were isolated from healthy control subjects and cryptococcal meningitis patients with the lymphocyte separation medium (Axis-Shield PoC AS, Oslo, Norway). The PBMC were stored in fetal cattle serum containing 10% DMSO at −70 °C.

Quantitative real-time PCR

RNA was isolated using RNeasy mini kits (Qiagen, Shanghai, China). cDNA was synthesized using 1 μg total RNA with random hexamers (Applied Biosystems, Shanghai), according to the manufacturer's instructions. All primers and probes (Table 1) were synthesized by Applied Biosystems (ABI). RT-PCR was performed in a 20 μl reaction volume, containing 1 μl of cDNA, 10 μl of PCR master mix, 1 μl of 20× mix and 8 μl of RNase-free water. The reaction was subjected to 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Relative target genes mRNA expression were calculated based on the differences between Ct values of target genes and Ct values of 18 s rRNA in paired samples.

Quantitation of the relative amount of target genes was carried out using the following equation:

$$\text{Target gene} = 2^{-(\Delta\Delta Ct)}$$

where ΔΔCt = (ΔCt, Q) − (ΔCt, C), (ΔCt, Q) represents the difference between Ct of target gene and Ct of housekeeping gene, (ΔCt, C) represents the difference between Ct of control gene and Ct of housekeeping gene. ΔCt values were presented as $\bar{x} \pm s$.All PCR assays were performed in triplicate.

Table 1
Sequences of RFQ-PCR probes and primers.

Gene	Role	Sequence
18 s rRNA	Sense primer	5′-ACATCCAAGGAAGGCAGCAG-3′
	Anti-sense primer	5′-TTCGTCACTACCTCCCCGG-3′
	Probe	FAM-CGCGCAAATTACCCACTCCCGA-TAMRA
BLyS	Sense primer	5′-GCAATCCAATCGGAGGGTAA-3′
	Anti-sense primer	5′-TCTGCATCTCTACCCCTACTGTACA-3′
	Probe	FAM-TGCCAGCAAACCTA-TAMRA
APRIL	Sense primer	5′-AGTCCTGCTTGCCAATTTCAG-3′
	Anti-sense primer	5′-GGCCGGGTGTGTTGGAA-3′
	Probe	FAM-CAGGGAGTAGTGCAGGC-TAMRA
TACI	Sense primer	5′-CTCAAGGCCCCGTCAAAGT-3′
	Anti-sense primer	5′-GCTTCCATCGCGTGATCCT-3′
	Probe	FAM-CGGCCAAGTCTTC-TAMRA
BCMA	Sense primer	5′-CTTAGCTGCCGCGAAGACA-3′
	Anti-sense primer	5′-TGAACTTCGCCTGCTTCGT-3′
	Probe	FAM-AGACAGCCCCCGTAAG-TAMRA
BAFF-R	Sense primer	5′-AGTTTGGTGTGCTTGCCTTTG-3′
	Anti-sense primer	5′-CCACCTTCAAGGGCTGTCA-3′
	Probe	FAM-CTTCAGACCTCACCATCT-TAMRA

Measurements of IgG, IgA, IgM, C3 and C4

Serum concentrations of IgG, IgA, IgM, Complement component 3 (C3) and C4 in each participant were determined in triplicate with the DADE BEHRING BN2 automatic protein analyzer. They were expressed as mean ± standard error.

ELISA

Commercial enzyme-linked immunosorbent assay (ELISA) kits, Human BLyS and APRIL ELISA (Abnova, American), were used for a quantitative determination of human APRIL in plasma samples using the manufacturer's protocols.

Growth of Cryptococcus in presence of PBMC

C. neoformans (5×10^3 CFUs) and 10^4 PBMC were coincubated in 150 μl medium containing 10% mixed human serum at 37 °C in the presence or absence of BLyS protein (10 μg/mL per well). *C. neoformans* was also grown in absence of PBMC as control. After 24 h incubation, PBMC incubated with *C. neoformans* were lysed by adding 0.1% TritonX-100. Serial dilutions were plated onto sabouraud dextrose agar at 24 h and 48 h post-culture. The colony forming units were counted 48 h post-inoculation. (Pretest results suggested that viability of fungus was not affected by TritonX-100.)

Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD) and tested with Student's *t*-test. Categorical data were expressed as numbers (%) and tested with Chi-square test. The correlation was tested with Pearson's correlation coefficients and presented with scatter plot. The analyses were performed using the SPSS 15.0 software package (SPSS Institute Inc., Chicago, USA). All statistical assessments were two-sided using a significance level of 0.05.

Results

Correlation of BLyS, APRIL and BLyS receptors with cryptococcal meningitis

The 62 subjects in this study included 30 patients in the CM group and 32 in the control group. Table 2 compared the clinical and laboratory data between the two groups. The average age for the 22 (35.48%) males and 40 (64.52%) females was 36.68 years (25–55 years). There were no statistically significant differences in age and gender between the CM patients and controls. The immunological markers exhibited several significant differences in CM patients. The B lymphocyte stimulator (BLyS) level was significantly lower in the CM

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