

The Anti-Inflammatory Properties of Interleukin 18 Binding Protein in Rheumatoid Arthritis

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Abstract

Objectives: Interleukin-18 binding protein (IL-18BP) is functioning as a natural anti-inflammatory and immunosuppressive molecule by neutralizing the effects of IL-18 during inflammation. This study aimed to identify the role of IL-18BP in the regulation of immune responses associated with the pathogenesis of RA.

Materials and Methods: 65 RA patients, 22 OA patients, and 40 sex and age matched healthy donors were enrolled in this study. Synovial specimens were obtained through synovectomy or arthroscopic procedures. SFMC and PBMC were prepared by using Ficoll-Hypaque separation procedure. Superarray analysis was used to measure the expression profile of immune-related genes in normal PBMC treated with recombinant human IL-18BP. The mRNA levels of Th1 and Th2 cytokines were measured by Real-time PCR, and the protein levels of IFN- γ , IL-4 were detected by ELISA.

Results: SuperArray analysis of immune related gene expression profile in normal PBMC treated with IL-18BP indicated decreases in the gene expression of IFN- γ and its regulatory molecules STAT-1 and STAT-2. This study pointed out that IL-18BP has additional anti-inflammatory property through downregulating the expression of IFN- γ and IL-12, at the same time, upregulating the expression of IL-4 and IL-10. Both IFN- γ and IL-12 could upregulate the mRNA and protein levels of IL-18BP in both the normal and RA subjects.

Conclusion: Our results demonstrated the importance of IL-18BP as an immune regulatory molecule and as a promising therapy for treating RA.

Key words: IL-18BP, Inflammation, Rheumatoid Arthritis, Osteoarthritis

Rheumatoid Arthritis (RA) is a chronic inflammatory disease that affects approximately 1% of the population in all parts of the world¹.

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Although the etiology and pathogenesis of RA is unknown, there is evidence indicating that T-cell mediated inflammation plays an important role in the rheumatoid synovitis. Several data suggest that T lymphocytes, in particular, Th1 cells, and array of proinflammatory cytokines and monokines are associated with inflammation and tissue damage in RA^{2, 3}. Certain cytokines such as IL-18 has been found to exhibit powerful Th1 promoting activities in synergy with IL-12 in RA⁴. Still the molecular mechanisms involved in the activation and perpetuation of inflammatory T cells in rheumatoid synovium are poorly understood.

Novick et al.⁵ identified IL-18BP as the natural inhibitor of IL-18. This gene product is an important potential candidate for neutralizing IL-18 in autoimmune diseases^{5, 6}. As such; it regulates IL-18-induced IFN- γ

production and consequently influences the Th1 and inflammatory responses. IL-18BP resembles the extracellular segment of a cytokine receptor in a single Ig domain. However, IL-18BP is a novel protein distinct from IL-1 and IL-18 receptor family members.

MATERIALS AND METHODS

A total of 65 Chinese patients with Rheumatoid Arthritis (RA), 22 patients with Osteoarthritis (OA), and 40 sex and age matched healthy donors were included in this study under the informed consent and the approval by the Institutional Medical Ethics Review Board of the Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences. Diagnosis of RA was defined according to the classification criteria of American College of Rheumatology⁷. All patients were from different parts of China, they referred to the Department of Rheumatology out clinic of Renji Hospital in Shanghai (China) in the period between 2005-2007.

Patients received any immunosuppressive or immunomodulatory drugs in the last two month preceding samples collection were excluded. RA patients group included 58 female and seven male with average age of 53±9.8 years. Synovial specimens were obtained through synovectomy or arthroscopic procedures that were performed for other medical indications. Synovial fluids (SF) were centrifuged at 350g for three minutes, and supernatants were collected and immediately stored at -80°C until use. Mononuclear cells were prepared by Ficoll-Hypaque separation (Amersham Biosciences) from SF, and blood specimens of RA patients and were immediately processed for cell culture.

Cell culture:

PBMC from healthy Chinese donors and RA patients were cultured in flat bottomed wells at 1X10⁶ cells/ml in RPMI stimulated with different concentration of IL-18BP_a (5 ng/ml, 50 ng/ml, 200 ng/ml). After 48 hrs, IFN- γ , IL-12, IL-4, IL-10 were measured by real time PCR (Q-PCR).

To study the adverse effect of blocking IL-18BP in RASF, normal PBMC was co-cultured with RA-SF pre-incubated with anti-human IL-18BP_a antibody in a dose-dependant manner for 48 hrs, IFN- γ mRNA was quantitated by Real-time PCR (Q-PCR). To see the Effect of Th1 and Th2 cytokines on IL-18BP_a production, PBMC (1X10⁶ cells/ml in RPMI 1640 medium with 10% fetal calf serum) from healthy donors (n=10) were cultured for 7 days in duplicate with or without IFN- γ (5ng/ml), IL-12 (1ng/ml), IL-4 (5ng/ml), and IL-10 (10ng/ml) or Anti-CD3 Antibody (1mg/ml).

cDNA array analysis: The expression analysis of selected cytokine and chemokine genes were examined using a commercially available cDNA array system containing 364 genes related to autoimmune and inflammatory response and 20 positive and negative control genes (SuperArray Bioscience Corporation, MD) according to the manufacturer's instructions. The gene list is given at the supplier's website (www.superarray.com). Briefly, PBMC from healthy individuals were treated with IL-18BP_a (0.2 mg/ml) for 48hrs. The gene expression profile was compared with untreated PBMCs under the same experimental condition. Three micrograms of total RNA were reversed transcribed into biotin-16-deoxy-UTP-labeled single strand cDNA by Moloney murine leukemia virus reverse transcriptase. After pre-hybridization, membranes were hybridized with biotin-labeled sample cDNA and incubated with alkaline-phosphatase-conjugated streptavidin. Chemiluminescence was visualized by autoradiography. The results were analyzed using GEArray Expression Analysis Suite (Version1.0) provided by SuperArray at its website. The relative expression of different genes was estimated by comparing signal intensity with that of average intensity of internal control genes. Data was expressed as ratio of significant change in gene expression (IL-18bp treated/untreated control).

ELISA:

The concentrations of the indicated IFN- γ and IL-4 cytokines were measured quantitatively using ELISA kits (Jingmei Biotech.) according to the manufacturer's procedure. SF, plasma or serum, along with the recombinant cytokines as standards, were diluted with PBS and added in duplicate wells. Plates were incubated for 2 hours and subsequently washed with PBS-Tween 20. Matched biotinylated detecting antibodies were added and incubated for 2 hours. After washing, avidin-conjugated HRP and 3,3',5,5'-tetramethyl benzidine were used for color development. Optical density was measured and cytokine concentrations were quantitated using microplate computer software (Bio-Rad Laboratories).

RNA extraction and Real time PCR:

Total RNA was isolated from cell pellets using an RNeasy Minikit (QIAGEN) with additional DNA digestion step (RNase-Free DNase Set; QIAGEN). First-strand cDNA synthesis was performed for each RNA sample using Sensiscript RT Kit (QIAGEN). Random hexamers were used to prime cDNA synthesis. SYBR green real-time PCR primers for human IL-18BP isoforms, selected cytokines, and GAPDH (housekeeping control) were designed using Primer Express software from Invitrogen (Table 1). The specificity and optimal primer concentration was tested. Thermocycler conditions included

an initial holding at 50°C for 2 minutes, then 95°C for 10 minutes; this was followed by a 2-step PCR program: 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. Data were collected and quantitatively analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (CT, cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (Δ CT) between the CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta$ CT) between the Δ CT values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as $2^{-\Delta\Delta$ CT.

Statistics: A student's t test was used to analyze the difference between the groups. Gene expression differences were analyzed by the Mann-Whitney U test. A value of $p < 0.05$ was considered statistically significant.

Table 1: Specific primers designed for real-time PCR (Q-PCR) analysis

Name	Primer	Sequence (5'-----3')	Product length (bp)
IFN- γ	FW	TCAGCTCTGCATCGTTTTGG	120
	RV	GTTCCATTATCCGCTACATCTGAA	
IL-12	FW	TGGAGTGCCAGGAGGACAGT	147
	RV	TCTTGGGTGGGTCAGGTTTG	
IL-10	FW	GTGATGCCCAAGCTGAGA	138
	RV	CACGGCCTTGCTCTTGTTTT	
IL-4	FW	CCACGGACACAAGTGCGATA	149
	RV	CCCTGCAGAAGGTTTCCTTCT	

FW = Forward; RV = Reverse. Bp = Base pair

Results

A total of 65 Chinese patients with RA, 22 patients with OA, and 40 sex and age matched healthy donors were included in this study. RA patients group included 58 females. The average age \pm standard deviation (SD) was

53 ± 9.8 years. Table 2 summarized the demographic and clinical data of the RA and OA patients referred to the outpatient clinic of Renji Hospital in Shanghai in the period between September 2005-2007.

Table 2: Demographic and clinical data of the RA and OA patients referred to the out clinic of Renji Hospital in Shanghai in the period between September 2005-2007.

Parameters	RA (n=65)	OA (n=22)
Age (mean \pm SD) in years	53 \pm 9.8	70 \pm 8.3
Disease duration (mean \pm SD) in years	10.6 \pm 6.6	11 \pm 7.8
Sex of (male/female)	7/58	4/18
ESR mean \pm SD mm/hour	44.9 \pm 28.9	26 \pm 13.6
Positive Rheumatoid factor (%)	85.1	NA
IgG Rheumatoid Factor \pm SD	532.1 \pm 923.9	NA
IgA Rheumatoid Factor \pm SD	454.0 \pm 608.1	NA
C-reactive protein \pm SD (mg/dl)	9.43 \pm 18.1	NA

NA, not available

Inhibition of IL-18BP α to the gene expression and production of Th1 cytokine:

First, we examined the expression profile of genes related to autoimmune and inflammatory response in normal subjects. A representative ex-vivo experiment representing the expression profile of selected genes of 20 positive and negative control genes in normal PBMC treated with IL-18BP α (GEArray S Series human autoimmune and inflammatory response gene array, SuperArray Bioscience corporation, MD). Notably, IFN- γ gene expression was decreased in treated PBMC compared to non treated one. In parallel to gamma interferon, STAT-1 and STAT-4 gene expression was also decreased as shown in Table 3.

From the aforementioned results, we hypothesized that IL-18BP α may have further anti-inflammatory property through decreasing the expression of Th1 cytokines presented in IFN- γ .

To this end, the in-vitro results in normal and RA-PBMC indicated the ability of IL-18BP α to inhibit the mRNA levels of IFN- γ and IL-12 (Figure 1 A1&A2) and as well as, it can augment the mRNA levels of Th2 cytokines represented in IL-10 and IL-4 (Figure 1 A3&A4). The above result was confirmed by doing ELISA for IFN- γ and IL-4 (Figure 1 B1&B2).

Next we addressed whether blocking IL-18BP α in RASF could reverse the results indicated in figure 2A. SF of RA patients were pre-incubated for 45 min with different concentration of human Anti-IL-18BP α antibody (R&D System). PBMC preparations were then exposed in-vitro to the indicated dilution (Figure 1 B3) of the treated SF for 48hours. PBMC cells were subsequently harvested and quantitatively analyzed for the mRNA expression of IFN- γ and IL-12. Altogether, these results highlighted the anti-inflammatory properties that IL-18BP α can play in RA synovium.

Table 3: Gene expression profile normal PBMC after treated with IL-18BP α Gene groups Genes altered Ratio

Description of increased genes	
Colony stimulating factor 2 (granulocyte-macrophage)	79.51
Toll-like receptor 9	5.02
Chemokine (C-X-C motif) ligand 6	4.82
Vascular endothelial growth factor	3.73
Mitogen-activated protein kinase 8	3.26
Forkhead Box P3	2.34
Interleukin 12B	2.14
Description of decreased genes	
Interferon, gamma	0.07
Suppressor of cytokine signaling 4	0.02
Signal transducer and activator of transcription 4 (STAT-4)	0.01
Chemokine (C-C motif) ligand 17	0.01
CAMP responsive element binding protein 1	0.01
Interleukin 9	0.01
Signal transducer and activator of transcription 1 (STAT-1)	0.01
Interleukin 19	0.00
Inducible T-cell co-stimulator	0.00
Sp3 transcription factor	0.00

Effect of Th1 and Th2 cytokines on IL-18BP α production:

Our result indicates that IL-18BP α can be induced by a variety of proinflammatory cytokines. PBMC from normal subjects was treated for 7 days with optimum concentration of IFN- γ (5mg/ml), IL-12 (1mg/ml), TNF- α (5 mg/ml), IL-4 (5mg/ml), and IL-10 (10 mg/ml) with or without anti-CD3 antibody stimulation (1mg/ml). As shown in figure 2 A1&A2, IL-18BP α expression was significantly up regulated by IFN- γ and IL-12 in a dose-dependant manner.

In conformity, IFN- γ , IL-12 could augment the production of IL-18BP α protein in-vitro in RA subjects (Figure 2 B1&B2).

Discussion:

An increase in IL-18 and its neutralizing inhibitor (IL-18BP) has been reported in RA serum compared with control⁸.

The cDNA array gene analysis in normal PBMC treated with recombinant IL-18BP α observed down regulation to IFN- γ gene production comparable with the signal transducer and activator of transcription 1 (STAT-1) and 4 (STAT-4), which are involved in IFN- γ production^{9,10}.

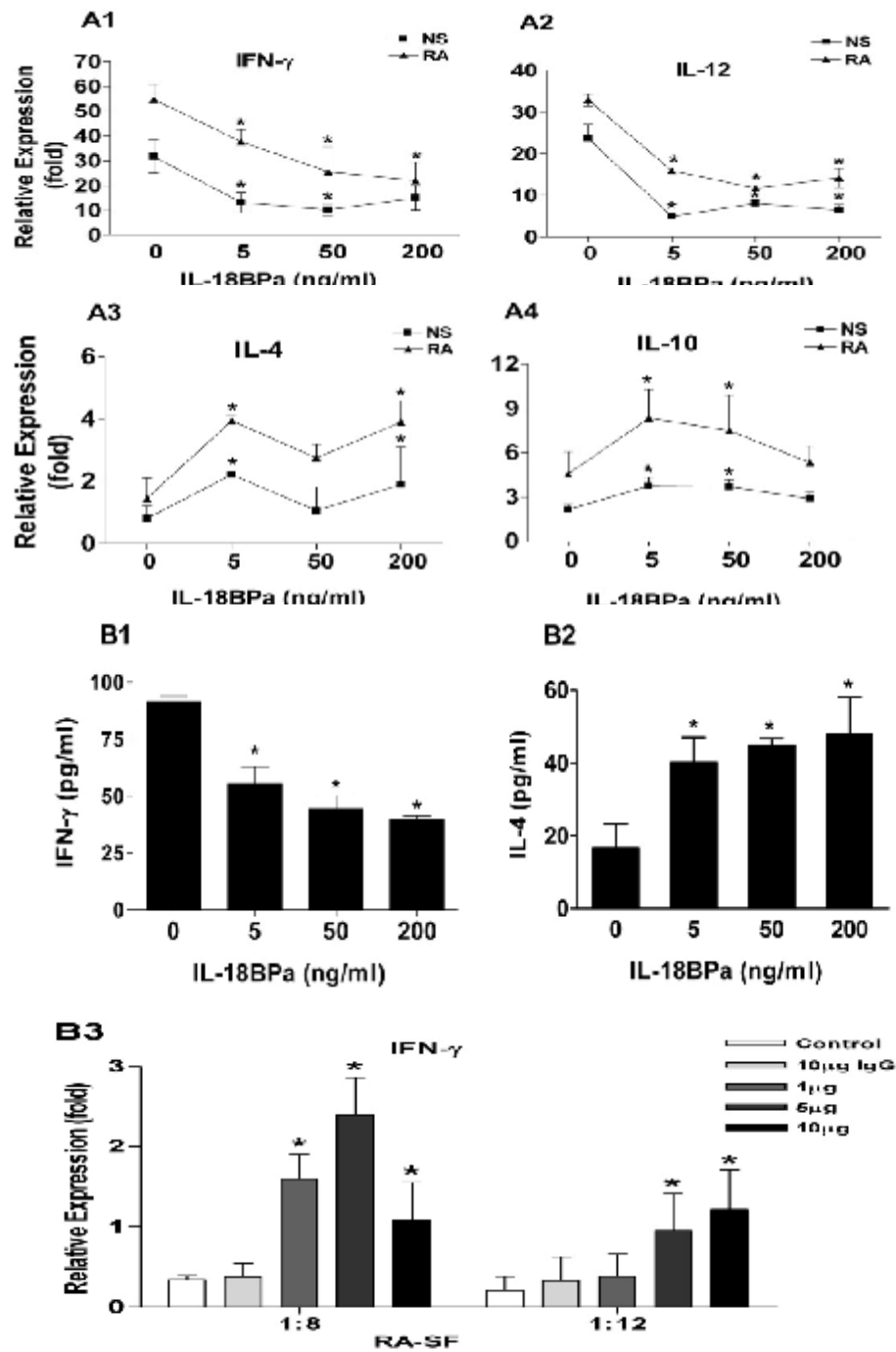


Figure 1: Regulatory effect of IL-18BPα on Th1 and Th2 mRNA transcripts and protein levels. The results are expressed as mean value of relative mRNA expression of transcript ± SEM. Single asterisk indicate significant different between the dose dependant concentration of IL-18BPα and the control (*P<0.05).

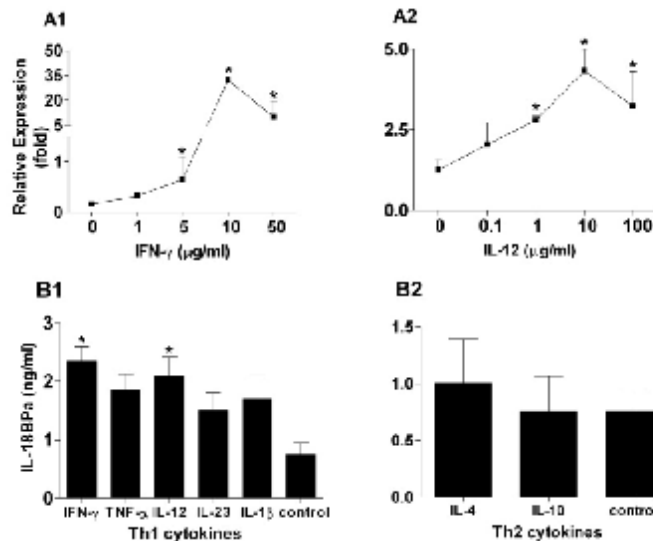


Figure 2: IL-18BP α production and mRNA expression by control peripheral blood mononuclear cells (PBMC). Bars showed the significant levels as mean \pm SD of independent experiments performed with supernatant and cells from different healthy donors. Asterisks indicate significant different (* P < 0.05).

This notion led us to go deep and see whether IL-18BP α may have an effect on the cytokines milieu in RA and normal subjects, taken into account a previous studies highlighted in a way or another, an indirect inhibition of IFN- γ by IL-18BP α ⁵. This in addition to the function of IL-18BP α as an early inhibitor to Th1 cytokines in animal models following IL-18BP α administration in CIA models, and abrogate circulating IFN- γ following LPS injection^{5,11}. Furthermore, the level of IFN- γ was found significantly reduced in serum of mice transgenic to IL-18BP compared to non transgenic ones¹². Also it has been found that the administration of IL-18BP α resulted in diminution of the local production of IFN- γ in patients with allergic contact dermatitis¹³. It could also reduces iNOS, TNF α and IFN- γ in Rats model administered with IL-18BP antibody used as control group in kidney allograft rejection¹⁴. In other compelling study, IL-18BP α was found down regulating IL-12 induced IFN- γ production in RA- and normal PBMCs¹⁵.

Compared to aforementioned results, here we observed an anti-inflammatory effect for IL-18BP α by reducing the release and expression of Th1 type cytokines represented in IFN- γ and IL-12. As well IL-18BP α has the ability to up regulated the expression and production of Th2 type cytokines represented in IL-4 and IL-10. Furthermore, blocking IL-18BP α in RA-SF increased the expression of IFN- γ in a dose-dependant manner.

Herein, the study found that both IFN- γ and IL-12 have the ability to enhance IL-18BP α production in a significant level, however, the other Th1 cytokines including IL-23, IL-1 β , and TNF- α can augment IL-18BP α production.

We also found that, the expression of IL-18BP α was significantly increased by IFN- γ and IL-12 in a dose-dependant manner. Likewise, IL-18BP α was strongly expressed by IL-12 mediated through IFN- γ in PBMC of healthy subjects¹⁶. However, only gamma interferon (IFN- γ) has the ability to up regulate the mRNA level of IL-18BP α in

synoviocytes¹⁷, endothelial cells and macrophages³, and in non leukocytes cells¹⁸. The discrepancy between our results and that reported by Kawashima and Miossec¹⁵, who found that IL-12 decreases the basal levels of IL-18BP α production by freshly isolated RA or control PBMCs, may be due to differences in assay condition, as well as, the different stimulation and application procedures used. In summary, in-vitro analysis indicated further anti-inflammatory properties to IL-18BP α on cytokines milieu, and IL-18BP α interaction with IFN- γ and IL-12 could represent a negative feedback mechanism upon established RA inflammation. Defining the roles of IL-18BP α and gaining a better understanding of the molecular function will facilitate the administration of IL-18BP α as promising therapy for treating RA and possibly other autoimmune diseases.

References:

1. Buckley CD. Treatment of rheumatoid arthritis. Science, medicine and future. *BMJ* 1997; 315: 236-8.
2. Banda, N. K.A. Vondracek, D. Kraus et al. Mechanisms of inhibition of collagen-induced arthritis by murine IL-18 binding protein. *J Immunol* 2003; 170: 2100.
3. Corbaz, A.T. ten Hove, S. Herren et al. IL-18-binding protein expression by endothelial cells and macrophages is up-regulated during active Crohn's disease. *J Immunol* 2002; 168: 3608.
4. Choy, E.H., and Panayi, G.S. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med* 2001; 344: 907-916.
5. Novick, D.S. H. Kim, G. Fantuzzi et al. Rubinstein. Interleukin-18 binding protein: novel modulator of the Th1 cytokine response. *Immunity* 1999; 10:127.
6. Szekanecz, Z., Kim, J., and Koch, A.E. Chemokines and chemokine receptors in rheumatoid arthritis. *Semin Immunol* 2003; 15: 15-21.
7. Arnet, F.C., Edworthy, S.M., Block, D.A et al. (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*; 31: 315-24.
8. Bresnihan B, Roux-Lombard P, Murphy E et al. Serum interleukin 18 and interleukin 18 binding protein in rheumatoid arthritis. *Ann Rheum Dis* 2002; 61(8): 726-9.
9. Afkarian M, Sedy JR, Yang J et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 2002; 3(6): 549-57.
10. Lawless VA, Zhang S, Ozes ON et al. STAT-4 regulates multiple components of IFN-gamma-inducing signaling pathways. *J Immunol* 2000; 165(12): 6803-8.
11. Plater-Zyberk C, Joosten LA, Helsen MM et al. Therapeutic effect of neutralizing endogenous IL-18 activity in the collagen-induced model of arthritis. *J Clin Invest* 2001; 108 (12): 1825-32.
12. Lonnemann G, Novick D, Rubinstein M et al. Interleukin-18, interleukin-18 binding protein and impaired production of interferon-gamma in chronic renal failure. *Clin Nephrol* 2003; 60(5): 327-34.
13. Plitz T, Saint-Mezard P, Satho M et al. IL-18 binding protein protects against contact hypersensitivity. *J Immunol* 2003; 171(3): 1164-71.
14. Wyburn K, Wu H, Chen G et al. Interleukin-18 affects local cytokine expression but does not impact on the development of kidney allograft rejection. *Am J Transplant* 2006; 6(11): 2612-21.
15. Kawashima M and Miossec P. Decreased response to IL-12 and IL-18 of peripheral blood cells in rheumatoid arthritis. *Arthritis Res Ther* 2004; 6: 39-45.
16. Veenstra KG, Jonak ZL, Trulli S et al. IL-12 induces monocyte IL-18 binding protein expression via IFN-gamma. *J Immunol* 2002; 168(5): 2282-7.
17. Moller B, Paulukat J, Nold M et al. Interferon-gamma induces expression of interleukin-18 binding protein in fibroblast-like synoviocytes. *Rheumatology* 2003; 42(3): 442-5.
18. Mühl H, Kämpfer H, Bosmann M et al. Interferon-gamma mediates gene expression of IL-18 binding protein in nonleukocytic cells. *Biochem Biophys Res Commun* 2000; 267(3): 960-3.