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*Research Article*

## The Presence of Interleukin 18 Binding Protein Isoforms in Chinese Patients with Rheumatoid Arthritis

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**Abstract: Objective:** Human IL-18BP gene encodes at least four distinct isoforms (IL18BP<sub>a-d</sub>) derived by alternative splicing. Their presence in RA local inflammation is not yet examined. This study aimed to determine the messenger transcript and protein levels of IL-18BP isoforms in patients with Rheumatoid Arthritis (RA). **Materials:** The study comprises 65 rheumatic patients, 22 Osteoarthritis (OA), and 40 sex and age matched normal controls (NC). Peripheral blood mononuclear cells (PBMCs) and synovial fluids mononuclear cells (SFMCs) were prepared by using Ficoll-Hypaque procedure. The expression and presence of different isoforms were determined by using real-time PCR and ELISA respectively. **Results:** IL-18BP messenger transcript has been extremely expressed in synovial fluids (SF) and synovial tissues (ST) of RA patients compared to OA patients ( $p < 0.001$ ). IL-18BP auto-antibodies were noticed in RA plasma and SF (41.7%; 37.9%), in OA-SF (9.0%), and in plasma of NC (4.0%). Comparable to different isoforms, isoform “c” showed significant local expression ( $p < 0.001$ ) in RA-SFMC and systematic expression ( $p < 0.001$ ) between RA- and NC-PBMCs, isoform “a” was least expressed. Isoform “c” and “d” proteins were solely detected by western blot in RA. **Conclusions:** This study emphasizes the local existence of isoform “c” and “d”, and the possible presence of autoantibodies against IL-18BP<sub>a</sub> in RA patients, which made a pea for further investigation, putting in place their actual role.

**Key words:** IL-18BP; Isoforms; Autoantibodies; Rheumatoid Arthritis

## 1. Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory disease that affects approximately 1% of the population in all parts of the world [1]. Although the etiology and pathogenesis of RA is unknown, there is evidence indicating that T-cell mediated inflammation plays an important role in rheumatoid synovitis. Novick et al. [2] identified IL-18BP<sub>a</sub> as the natural inhibitor of IL-18. This gene product is an important potential candidate for neutralizing IL-18 in autoimmune diseases [2,3]. There are four human (hIL-18BP<sub>a/b/c/d</sub>) and two mouse (mIL-18BP<sub>c/d</sub>) isoforms resulting from mRNA splicing. These were found in various cDNA libraries with hIL-18BP<sub>a</sub> cDNA as the most abundant clone. The inhibition of IL-18 was tested for the 4 purified isoforms by using different molar ratio of IL-18BP, complete inhibition of IL-18 activity on natural killer cells was observed at a twofold molar excess, while at equimolar ratios, the inhibition by IL18BP<sub>a</sub> was approximately 50%. Equimolar and molar excess of two of human IL18BP<sub>c</sub> exhibited similar potency as IL18BP<sub>a</sub> [4]. The human isoforms IL-18BP<sub>a</sub> and IL-18BP<sub>c</sub> exhibited the greatest affinity for IL-18 with dissociation constants K<sub>d</sub> of 399 pM and K<sub>d</sub> of 2.94 nM, respectively and they inhibited 50% IL-18 at equimolar ratio, a notion explain unusual low molar ratio to observe inhibition of biological activity in a 24 hour bioassay, comparable to soluble receptors such as the IL-1 receptor type I and II and the TNF receptor p55 and p75, the molar excess required to inhibit 50% of biological activity of the respective ligands is at least five fold [5,6]. On contrary, the other two isoform IL-18BP<sub>b</sub> and IL-18BP<sub>d</sub> only possess an incomplete immunoglobulin domain. By virtue of this structural difference these variants are unable to neutralize IL-18 [4]. In this study, we detected and autoantibodies against IL-18BP and we observed the expression of isoform 'd' in addition to isoform 'c' in RA synovium. Quantitative comparison between isoforms is needed to validate the exact role of inactive isoform 'd'.

## 2. Methodology

### 2.1. Patients

This is a hospital based, case control study, encompasses 65 patients (58 females, 7 males; age  $53 \pm 9.8$  years) clinically confirmed having RA, 22 patients (18 females, 4 males; age  $70 \pm 8.3$  years) suffering from OA, and 40 normal (36 females, 4 males,  $52.6 \pm 8.7$  years) control (NC). Details of demographic data and laboratory profile are presented in Table 1. The study cases referred to the out clinic of Renji Hospital in Shanghai (China) within the period from 2011 to 2013. They are diagnosed at the Department of Rheumatology based on the classification criteria of the American College of Rheumatology [7]. Exclusion criteria applied to cases under prescription for any

immunosuppressive and or immunomodulatory drugs back dated for two months as minimum period before sampling. Heparanized blood, in addition to synovial fluids (SF) and tissues (ST) which were collected through synovectomy and arthroscopic operations has been performed for other medical purposes. Synovial fluids were centrifuged for 3 minutes at 350 g, supernatants were collected and immediately stored at  $-80^{\circ}\text{C}$  until use. All participants were informed with the study purpose and they consent to participate, in addition to a study approval obtained from the Institutional Medical Ethics Review Board of Shanghai Institutes of Biological Sciences (SIBS), and Chinese Academy of Sciences (CAS).

**Table 1. 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 2011-2013.**

Parameters	RA (n = 65)	OA (n = 22)
Age, mean years	53 ± 9.8	70 ± 8.3
Disease duration, mean years	10.6 ± 6.6	11 ± 7.8
Sex of (male/female)	7/58	4/18
ESR mean mm/hour	44.9 ± 28.9	26 ± 13.6
Positive Rheumatoid factor (%)	85.1	NA
IgG Rheumatoid Factor	532.1 ± 923.9	NA
IgA Rheumatoid Factor	454.0 ± 608.1	NA
C-reactive protein (mg/dL)	19.43 ± 18.1	NA

NA, not available

## 2.2. Cell Culture

PBMCs from peripheral blood and SF were isolated by using 7.5 mL Ficoll-Hypaque density gradient centrifugation at 2000 rpm without break for 20 min at  $20^{\circ}\text{C}$ , washed with RPMI-1640 medium (complete medium) supplemented with 25 mM HEPES buffer and L-Glutamine, in addition to 10% heat-inactivated fetal calf serum (FCS), 100 U/mL Penicillin, and 100  $\mu\text{g}/\text{mL}$  Streptomycin (all purchased from GIBCO BRL Life Technologies, USA). The white belt generated as a result of centrifugation was diluted (10 $\times$ ) with PBS, counted by hemocytometer. Cells were adjusted to either  $1 \times 10^6$  or  $0.2 \times 10^6$  cells/ml and cultured in a 24 well plate (Becon Dickenson Labware Europe), or in 96-flat bottom polypropylene well plates (Becon Dickenson Labware Europe) respectively. Cells were harvested and supernatants were collected from different plates for determination of IL-18BP by using ELISA and real-time PCR.

## 2.3. Quantitative measures of IL-18BP and IL-18BP auto-antibodies

Quantitative measure of IL-18BP from culture supernatants was conducted according to manufacturer protocol provided with ELISA kits (R&D Systems Incorporation). In brief, 2  $\mu\text{g}/\text{mL}$

PBS of purified recombinant human IL-18BP (human IgG1/Fc chimera; R&D system) is used for capturing IL-18BP auto-antibodies. Plates were coated overnight at 4 °C, followed by adding 10% (w/v) FBS for 1 hour then washed. Samples (serum, plasma, and SF) were added in duplicate to plates after dilution with PBS. Plates were incubated for 2 hours and subsequently washed with PBS-Tween 20. Secondary biotinylated antibodies were added and incubated for 2 hours. After washing, avidin-conjugated HRP and 3,3',5,5'-tetramethyl benzidine were added for color development. Optical density was measured and cytokine concentrations were determined using microplate computer software (Bio-Rad Laboratories).

#### 2.4. RT-PCR analysis

Total RNA isolation was performed with RNeasy kits according to the protocol from the manufacturer (Qiagen). RNA purity was quantified spectrophotometrically by 260 nm absorbance and checked by optical density (OD) of 260 nm and 280 nm and by using agarose gel stained with ethidium bromide. cDNA synthesis was made by using Sensiscript RT kit (Qiagen) using RNA as a template. Synthesis was carried out in a 20 µL reaction volume containing 4 µg of total RNA, and random hexamers (50 µM) were used as internal enzyme start sites. PCR reaction takes place for 60 minutes at 37 °C. Subsequently the enzyme “Sensiscript reverse transcriptase” was inactivated by heating the reaction mixture to 93 °C for 5 minutes followed by rapid cooling in 4 °C, and stored in the same degree.

#### 2.5. Real-time PCR

Primers for IL-18BP (published sequence [AF110799](#)) which did not distinguish different isoforms, and primers specific for IL-18BP isoforms (a, b, c, and d; published sequence [AF110799](#), [AF110800](#), [AF110801](#), and [AF215907](#) respectively), and GAPDH (published sequence [M33197](#)) were designed (Table 2) using the primer express software from Invitrogen Biotechnology Co., Ltd. Clustal W (1.83) program applied for multiple sequence alignment of IL-18BP isoforms. SYBR Green real-time PCR was performed on the ABI prism-sequence detection system 7900 HT (Applied Biosystems) as follows: initial holding at 50 °C for 2 min, 95 °C for 10 minutes; followed by a 2-step PCR program: 95 °C for 15 seconds, 60 °C for 60 seconds comprises 40 cycles. The GAPDH gene was used as an endogenous control to normalize the 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 ( $\Delta$ CT) between the CT value of the sample for the target gene and the mean CT value of the endogenous control (GAPDH). Relative expression was calculated as the difference ( $\Delta\Delta$ CT)

between the  $\Delta$ CT values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as  $2^{-\Delta\Delta CT}$ .

**Table 2. Specific primers designed for real-time PCR (Q-PCR) analysis.**

Name	Primer	Sequence (5'-----3')	Product length (bp)
IL-18BP	FW	ACC TCC CAG GCC GAC TG	342
	RV	CCT TGC ACA GCT GCG TAC C	
IL-18BP <sub>a</sub>	FW	CAG CTC TGG GCT GGG CTG AG	203
	RV	GGG GTG TGT TGC GCA TCC AC	
IL-18BP <sub>b</sub>	FW	TGG AAG TGC CAC TGA GCT G	107
	RV	CCC TGT GCT GAG TCT TAA CCC	
IL-18BP <sub>c</sub>	FW	CCC AGC ATT CCT CAA GGT CA	120
	RV	AAG GCA GAC AAG GAT CAG GC	
IL-18BP <sub>d</sub>	FW	AAC GTG GGA GCA CAG GCT GG	212
	RV	GAG GGG GTG TGT TGC GCA TC	
GAPDH	FW	GTG AAG GTC GGA GTC AAC	150
	RV	TGA GGT CAA TGA AGG GGT	

FW = Forward; RV = Reverse; BP = Base pair

## 2.6. Western Blot

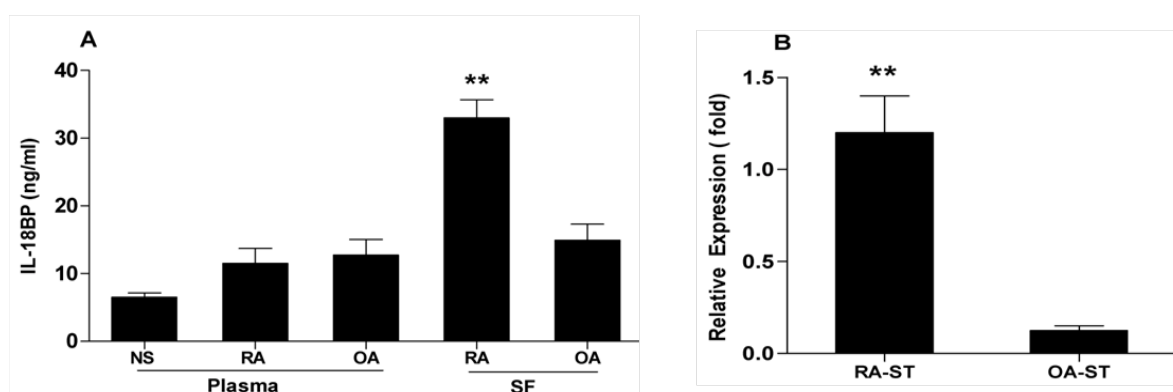
For IL-18BP isoforms detection, SDS-PAGE was used to separate RA-SFMC at a final concentration of 12% and transferred onto a nitrocellulose membrane (BIO-RAD, USA). Membranes were blocked for 1 hour at 37 °C with 5% TTBS-defatted milk powder and then incubated for 1 hour at room temperature with 0.1 µg/ml goat anti-human IL-18BP antibody (R&D System). Membranes then washed extensively (3 times for 5 minutes each) with TTBS before incubation with donkey anti-goat IgG HRP-conjugated secondary antibody (Jackson ImmunoResearch). After repeated washing with TTBS, the membranes were incubated in a substrate solution (Pierce) containing luminol for horseradish peroxidase (HRPO) reagent for 5 minutes, and then exposed to ECL hyperfilm (Kodak Film), and after 30 minutes development, the film was applied for autoradiography (Sigma-Aldrich).

## 2.7. Statistical analysis

Experiments were performed in duplicate and triplicate. Results were expressed as the mean  $\pm$  SD of the indicated number of samples. Within group comparisons were analyzed by Student's paired *t*-test. An ANOVA and Mann-Whitney *U* test corrected by Bonferroni method were used to determine the difference between different groups. Gene expression differences were analyzed by the Mann-Whitney *U* test. *p* value less than 0.05 considered significant.

### 3. Results

Fifty six RA patients, their mean age  $53 \pm 9.8$ , and 22 OA patients their mean age  $70 \pm 8.3$ . The male to female ration was around 1:7 in both cases. The overall laboratory investigations for RA patients were presented in (Table 1). Plasma, and synovial fluids (SF), were processed and analyzed for determination of IL-18BP concentration which was significantly high ( $p < 0.001$ ) in RA-SF (Figure 1a). IL-18BP expression was found to be highly expressed ( $p < 0.001$ ) in synovial tissues (ST) of RA patients compared to OA (Figure 1b). The high expression of IL-18BP in RA synovium accompanied its high levels in RA serum [8], nevertheless disease subsistence let us to postulate the presence of proteins or auto antibodies that may hinder the given proinflammatory effect of IL-18BP.

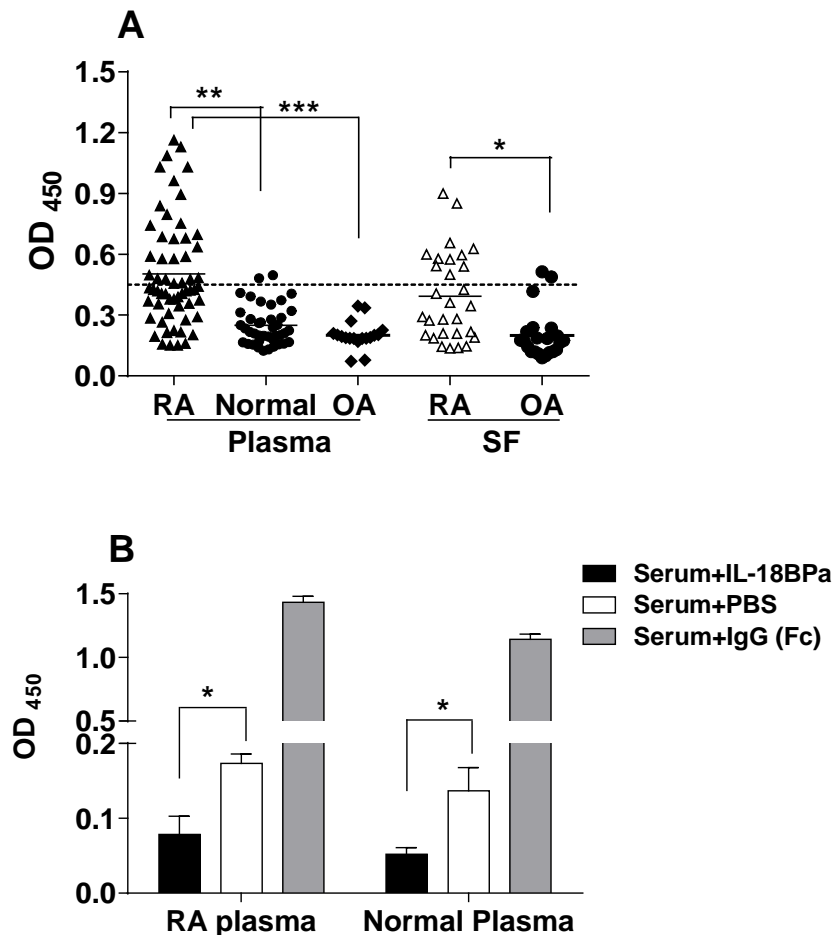


**Figure 1. IL-18BP mRNA and protein concentration in RA vs. OA synovium.** (A) The IL-18BP protein concentration was measured by ELISA in 65 paired plasma and SF in RA and 22 OA patients. A panel of 40 plasma samples from healthy subjects was included as control. (B) RNA was extracted from ST of RA ( $n = 20$ ) and OA ( $n = 10$ ) for real time PCR. IL-18BP expression was normalized to endogenously expressed GAPDH in the same samples. Relative expression was calculated as the difference between the ( $\Delta Ct$ ) values of the test sample and of the endogenous control (GAPDH). Results show the mean and SD of independent experiments performed in all study donors. Asterisks indicate statistically significant difference between the two groups (\*\* $p < 0.001$ ).

In an attempt to demonstrate the presence of auto-antibody against IL-18BP in plasma and SF of RA and OA patients. SF and plasma from 45 RA patients, 20 OA patients, and 25 NC were examined by a specific sandwich ELISA using plate coated with recombinant human IL-18BP (human IgG1 Fc), auto-antibodies binding to IL-18BP were examined by goat anti-human IgG mAb with fragment specific for the F(ab)2 to prevent its binding to the Fc part of IL-18BP.

As shown in Figure 2a, an auto-antibodies against IL-18BP were detected in plasma of 25 out of 60 (41.7%) and in SF of 11 out of 29 (37.9%) RA patients, while the auto-antibodies were detected in 2 out of 22 (9.0%) OA-SF, and were marginally positive in 2 out of 40 (5.0%) plasma of NC. ELISA

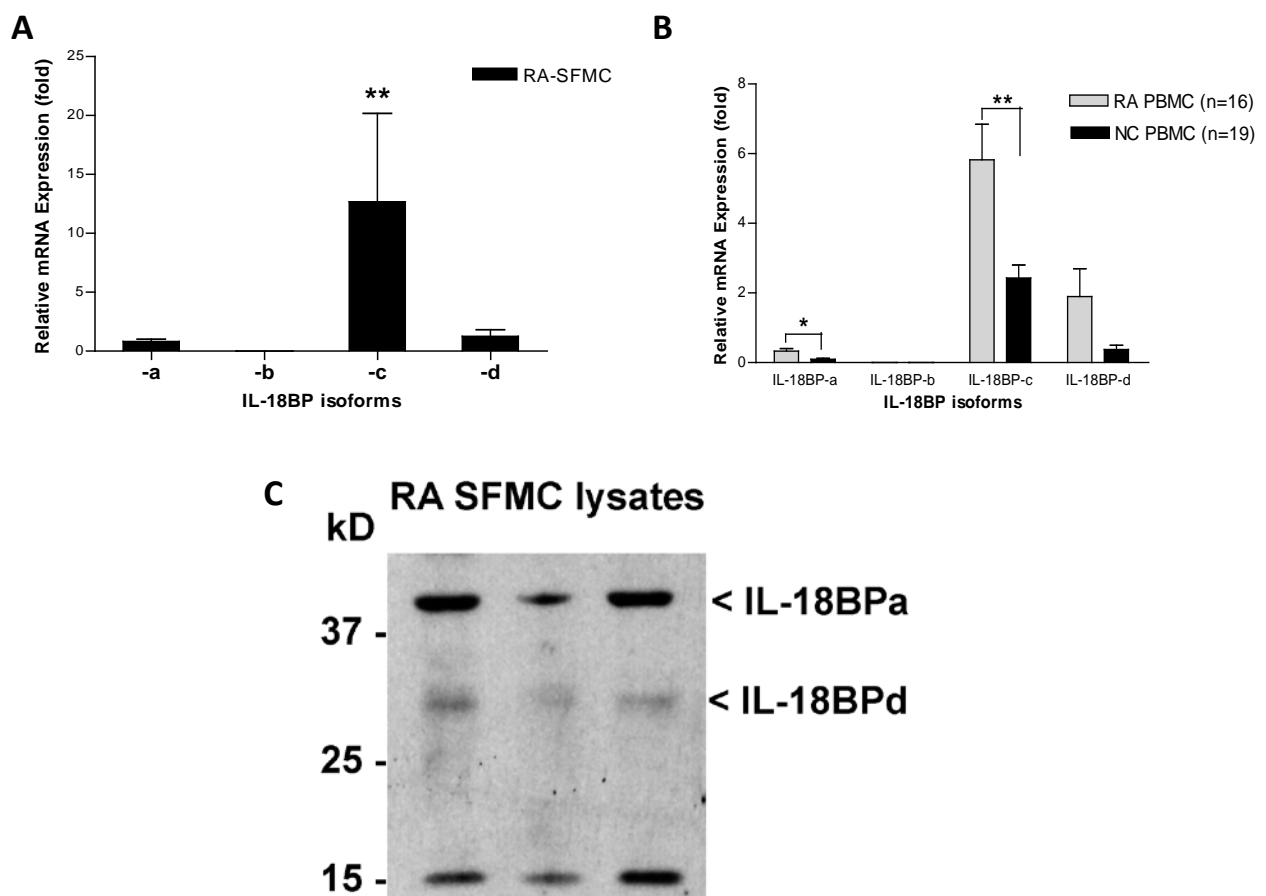
specificity to human IL-18BP<sub>a</sub> is evaluated by the pre-incubation of plasma from NC and RA subjects with soluble IL-18BP<sub>a</sub>, rather than control human IgG (Figure 2b). We used 0.443 OD<sub>450</sub> as a cut-off point based on the mean (0.249) + 2SD (0.0972) of values with plasma from 40 normal donors at 1:2000 dilutions.



**Figure 2. Detection of IL-18BP<sub>a</sub> auto-antibody in RA plasma and SF.** (A) Diluted plasma and SF of RA and OA patients and plasma of healthy controls were tested for binding to IL-18BP<sub>a</sub> by ELISA. Samples with OD<sub>450</sub> > 0.443 were considered positive. (B) To examine the specificity of the RA plasma binding to IL-18BP<sub>a</sub>, diluted plasma were pre incubated with PBS, 2µg/mL of soluble IL-18BP<sub>a</sub> or control IgG (hIgG1). Results are shown as mean and standards deviation (mean ± SD) as representative data of one of two independent experiments. Asterisks indicate significant difference between experimental groups (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.000).

The human IL-18BP gene encodes at least four distinct isoforms (IL-18BP<sub>a</sub>-d), which are derived by alternative splicing. It was known that IL-18BP isoforms ‘a’ and ‘c’ neutralize the biological activity of IL-18, whereas ‘b’ and ‘d’ do not [4]. In Crohn’s disease, isoform ‘a’, ‘c’ and ‘d’ were expressed [9]. It

is presently unknown whether isoforms 'b', 'c', and 'd' were expressed and/or secreted in RA patients. To see if these isoforms can be expressed in tissues of RA compared to NC, primers specific to each isoform was designed, RNA was extracted, and cDNA was synthesized as described earlier. Q-PCR was performed for the different isoforms using the GAPDH as an internal control. Our results revealed that, IL-18BPc was predominant and significantly overexpressed in RA-SFMCs (Figure 3a) and in RA-PBMCs compared with normal PBMCs, followed by isoform 'd' and 'a' (Figure 3b). Isoform 'a' was the least expressed isoform in RA and normal cells. There is no expression for isoform 'b' in different cell types. We cloned the target genes for each isoform and sequence it in order to confirm sequence specific product for each isoform, as well as to find out if there is any mutation for worthy (Data not shown). We successfully detected isoform 'ac and 'd' in RA-SFMC by western blot (Figure 3c).



**Figure 3. IL-18BPc is least expressed in both RA-SFMC and RA-PBMC while IL-18BPc is predominant.** (A) RNA was extracted from RA-SFMC and a real-time PCR was performed to cDNA for each isoform. (B) The mRNA expression of each isoform was analysed by preparing cDNA from RA and normal PBMCs. (C) prepared RA-SFMCs were collected from SF, SDS at a final concentration of 12% was used to separate isoforms proteins. Detection of the signals, calculation of threshold cycles (Ct values), and further analysis of these data were performed by the sequence detector software (SDS 2.0). All



results for IL-18BP isoforms were normalized to GAPDH. Asterisks indicate statistically significant difference (\*\* $p < 0.001$ ).

#### 4. Discussion

It has been stated that, serum from RA patients characterized by high levels of the proinflammatory cytokine IL-18 and its neutralizing inhibitor (IL-18BP<sub>a</sub>) [7,8], and in Crohn's diseases [9]. Nevertheless, no clear data indicates the levels and the expression of the neutralizing inhibitor in RA synovium. In this study we reported the significant expression of IL-18BP in RA local inflammatory areas (synovium) compared with OA patients.

Owing to our result and on previous aforementioned studies, the high levels of IL-18BP as a neutralizing inhibitor to IL-18, which is supposed to be high in response to inflammation, raised the possibility of finding proteins or auto-antibodies characterized by high similarity or specificity render it capable to influence or specifically binds to IL-18BP, thus hinder its biological activity. For instance, and supporting to our notion, it has been stated that, IL-1H4 an IL-1 related protein has had a high degree of similarity to IL-18 [10,11], it was there for possible that IL-1H4 could bind IL-18BP. Accordingly, we found a significant increase in IL-18BP<sub>a</sub> auto-antibody in plasma and SF from RA patients compared with OA and NC. The specificity of these auto-antibodies to IL-18BP<sub>a</sub> was determined by pre-incubated the samples with soluble rHIL-18BP<sub>a</sub> antibodies and by using positive and negative controls. Further studies are needed to unravel the exact role the autoantibodies may confer against IL-18, IL-18BP, and/or the pathogenesis of RA.

It has been found that at equimolar ratio, both IL-18BP<sub>a</sub> and IL-18BP<sub>c</sub> has the potency to neutralize 50% of IL-18 activity, however, IL-18BP<sub>c</sub> affinity for IL-18 was approximately 10 fold less than IL-18BP<sub>a</sub> [4]. In contrast, there was no inhibition to IL-18 activity by IL-18BP<sub>b</sub> and IL-18BP<sub>d</sub>, probably because they lack the Ig domain that possesses the essential binding requirement to IL-18. Corbaz and his co-workers [9] had analyzed the expression of the four IL-18BP isoforms (a, b, c, & d) in Crohn's disease and in normal subjects. They found that, unbound IL-18BP isoform 'a' & 'c' and the inactive isoform 'd' were present while isoform 'b' was not detected, their bands are (42 kDa, 40 kDa, and 35 kDa) respectively. Nevertheless, there is no study indicates the expression of these isoforms in RA patients. This study highlighted the expression of different IL-18BP isoforms (a–d) in vivo in RA patients. We found significant expression to IL-18BP proteins and its RNA transcripts in synovial fluids of RA patients as compared to OA patients. Refer to the RNA transcripts of IL-18BP isoforms, IL-18BP<sub>c</sub> transcript was predominant in RA-SFMC and RA-PBMC compared with the NC-PBMC, followed by isoform 'd' which was also remarkably higher in RA patients compared to NC. The in vivo expression of isoform 'd' which is to some extent equal to the expression of isoform 'a' in RA synovium require more attention despite previous studies approved its inability to neutralize IL-18. Comparable to our results, Corbaz et al [9], detected high levels of isoform 'c', 'd', and isoform 'a' in all the patients with Crohn's disease, however, they were unable to detect isoform 'b'. In this study, western blot analysis showed the

existence of isoform 'c' & 'd' with a molecular mass of 40 and 35 kDa, respectively. We couldn't apply quantification analysis to isoforms 'd' and 'c' due to unavailable reagents so far. Although, we successfully sequenced (complete CDS) isoform 'a' and isoform 'd' but not isoform 'c' neither from the PCR product nor in sequencing analysis (data not shown).

Taken together, our results imply the presence of IL-18BP auto-antibodies, in addition to the presence of isoform 'c' and 'd' in RA synovial which was found to be contradictory to the data indicated by other studies. Further studies are needed to quantify and then investigate the exact role of isoform 'c', and 'd' in RA progression.

### **Acknowledgment**

This study was supported by TWAS/CAS fellowship organization. We thank Dr. Wenxin Zheng, Xi Chen for technical assistant.

### **Conflict of Interest**

The authors declare no conflict of interest.

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