The expression of P2X7 receptors on peripheral blood mononuclear cells in patients with primary Sjögren's syndrome and its correlation with anxiety and depression

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Abstract

Objective

To study surface expression of P2X7 receptors (P2X7R) on peripheral blood mononuclear cells (PBMC) in patients with primary Sjögren's syndrome (pSS), and its correlation with anxiety and/or depression.

Methods

The Hamilton Anxiety Scale (HAMA) and Hamilton Rating Scale for Depression (HRSD) were used to assess 31 patients with pSS. P2X7R expression on the surface of $CD14^+$ and $CD14^-$ PBMC, with or without ATP stimulation, was measured by flow cytometry. IL-1β and IL-6 levels in blood plasma and supernatant after ATP stimulation were measured by ELISA. Nineteen patients with rheumatoid arthritis (RA), 18 patients with anxiety and/or depression, and 20 healthy cases were used as controls.

Results

P2X7R expression was detected in all subjects. Compared with no ATP stimulation, significant up-regulation of P2X7R expression on $CD14^+$ PBMC was observed after ATP stimulation in the pSS group only ($p=0.001$), while on $CD14^-$ PBMC there was significant up-regulation in both the pSS ($p<0.001$) and anxiety/depression ($p=0.003$) groups. After ATP stimulation, P2X7R expression on $CD14^+$ PBMC in the pSS group was significantly higher than the RA group ($p=0.044$), anxiety/depression group ($p=0.004$) and healthy controls ($p=0.002$). Moreover, in the pSS group, P2X7R expression on $CD14^+$ PBMC was significantly positively correlated to IL-1β supernatant levels ($r=0.447$, $p=0.025$). Overall, there were 45.2% (14/31) patients with anxiety and 32.3% (10/31) with depression, in the pSS group. P2X7R expression on $CD14^-$ PBMC was significantly positively correlated to scores of anxiety ($r=0.344$, $p=0.030$) and depression ($r=0.319$, $p=0.045$).

Conclusion

Surface expression of P2X7R on PBMC in patients with pSS was significantly higher than controls, suggesting P2X7R may contribute to the complex pathogenesis of pSS and also anxiety and/or depression.

Key words

Sjögren's syndrome, P2X7 receptor, anxiety, depression

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Introduction

Primary Sjögren's syndrome (pSS) is a chronic systemic autoimmune disease affecting approximately 0.5% of the population worldwide. Although pSS occurs within both genders in all age groups, the average age of onset in women is after the menopause, with a male:female ratio of 1:9–1:20. The pathogenesis of pSS is not well understood. Clinical observations find that pSS patients often have psychiatric symptoms such as anxiety and depression. In 1988, Angelopoulos *et al*., (1) observed that pSS patients present mainly with depression, somatisation, anxiety and obsessive-compulsive symptoms. Drosos *et al*., (2) assessed psychiatric symptoms using the hostility and direction of hostility questionnaire (HDHQ), and symptom checklist-90 (SCL-90). Compared with cancer patients and healthy controls, SS patients scored high on introverted hostility, paranoid ideation, somatisation and obsessive compulsive symptoms. Valtysdottir *et al*., (3) found using the Hospital Anxiety and Depression Scale, that pSS patients had significantly higher scoring rates for "possible" clinical anxiety (48%) and depression (32%) compared with rheumatoid arthritis (RA) groups. Stevenson *et al*., (4) reported that early recognition and appropriate intervention is essential to reduce the negative impact of depression on the quality of life for pSS patients. Barbara Segal *et al*., (5) states that depression is one of the substantial unmet health needs for pSS patients. Overall, these findings indicate that there may be a link between pSS and psychiatric symptoms, with the underlying mechanism potentially involved in common pathogenesis of two distinct disorders.

Purinergic receptors are classified as P1 and P2 receptors, with adenosine acting on P1 receptors and ATP on P2 receptors. In turn, P2 receptors are further sub-classified into P2X and P2Y receptors. P2X receptors are ionotropic ligand-gated non-selective cation channel receptors, while P2Y receptors are G-protein coupled receptors. Currently, there are seven P2X subtypes (6). The P2X7 receptor (P2X7R) is a distinct member of the P2X subclass, with its downstream signaling coupled to pro-inflammatory cascades. The P2X7R is mainly expressed on macrophages/monocytes, microglia and certain lymphocytes, and plays a key role in regulating cell survival and release of certain pro-inflammatory molecules, including IL-1β and IL-6 (7, 8). Moreover, through regulation of pro-inflammatory cytokine release and cell death, P2X7R activity may be involved in the pathophysiology of neuropsychiatric disorders (including anxiety and depression (9)), and autoimmune disease (including systemic lupus erythematosus (10) and RA (11)).

With regards pSS, studies have implied that P2X7R may play an important role in SS pathogenesis. Baldini *et al*., found that P2X7R expression is significantly higher in salivary glands from pSS individuals, suggesting involvement of the P2X7R-inflammasome-caspase-1-IL-18 axis in development of pSS exocrinopathy (12).

Auto-antibodies specific for alpha-fodrin fragments (a cytoskeletal protein thought to act as an autoantigen in SS development) are found in tissues from SS patients. Hwang *et al*., used Par C5 cells to examine the role of P2X7R in apoptosis, concluding that P2X7R plays an important role in apoptosis and alpha-fodrin degradation in salivary epithelial cells. Therefore, P2X7R may be involved in SS pathogenesis via the mechanism of apoptosis (13).

In vitro studies using submandibular gland cell aggregates isolated from mice, indicate that P2X7R activation with ATP or BzATP stimulates cleavage and release of alpha-fodrin (14). Thus, P2X7R may represent a critical communication link between the nervous and immune systems.

In the present study, we examined surface expression of P2X7R in peripheral blood mononuclear cells (PBMC) and the production of pro-inflammatory mediators in whole blood after ATP stimulation, in pSS patients, and determined its correlation with anxiety and/or depression.

Materials and methods

Subjects

A total of 31 pSS patients (29 female and 2 male, mean age 53±14; 5 new

cases), 19 RA patients (16 female and 3 male, mean age 61±10), 18 anxiety and depression patients (16 female and 2 male, mean age 51±9), and 20 healthy volunteers (18 female and 2 male, mean age 51±11) were enrolled in the study. Patients attended the Rheumatology Department at Ningbo no. 2 Hospital, and the Psychological Department at Ningbo Kangning Hospital, in China. The pSS patients were diagnosed according to the Revised International Classification Criteria for Sjögren's Syndrome (2002), and RA patients according to the ACR/EULAR Rheumatoid Arthritis Classification Criteria (2010) but without secondary Sjögren's syndrome. Patients with anxiety and depression were excluded from other disease diagnoses, including autoimmune disease. Healthy volunteers were healthy medical staff (including retirees) from Ningbo No.2 Hospital. Informed consent was obtained from each subject. The Medical Research and Ethics Committee (MREC) at Ningbo No.2 Hospital approved the study (Table I).

Table I. Clinical and routine biochemical characteristics of 31 patients with primary Sjögren's syndrome.

Age (years)	53 (23~81)
Female sex	29 (93.5)
Disease duration (months)	67 (35~120)
Extraglandular affectation	11 (35.5)
Subjective ocular dryness	28 (90.3)
Subjective oral dryness	24 (77.4)
Salivary gland swelling	4 (12.9)
Schirmer test 55 mm/5 min	31 (100)
Erythrocyte sedimentation rate (mm/h)	36 ± 24.53
C-reactive protein (mg/dL)	15.1 ± 27.1
Rheumatoid factor (UI/L)	43.2 ± 72.23
Serum immunoglobulin G concentration(mg/dL)	1583 ± 504
Serum immunoglobulin A concentration(mg/dL)	287 ± 88
Serum immunoglobulin M concentration(mg/dL)	183 ± 54
C3 (mg/dL)	82.9 ± 23.3
C4 (mg/dL)	16.1 ± 7.3
Presence of anti-nuclear antibodies	29 (93.5)
Presence of Anti-Ro	29 (93.5)
Presence of Anti-La	9 (29.0)

Psychological assessment

The Hamilton Anxiety Scale (HAMA) and Hamilton Rating Scale for Depression (HRSD; 24 items) were used to assess the presence and degree of a patient's anxiety and depression. The same two independent qualified and experienced clinicians choose the possible response to each question by interviewing the patient and observing the patient's symptoms. The average score provides the result (scorer reliability >0.90). A HAMA total score >14 is considered indicative of anxiety, while a HRSD total score >20 is considered indicative of depression, according to the Chinese norm.

Peripheral blood mononuclear cell (PBMC) preparation

Peripheral venous blood was collected from each subject in heparin-containing vacuum tubes. PBMC were isolated by density centrifugation on Ficoll-Hypaque columns (GE Healthcare, USA). Buffy coats were harvested, and cells washed by centrifugation at 300×g for 5 min in Hanks balanced salt solution (HBSS; Hyclone, USA), and maintained in RPMI-1640 medium (Hyclone) supplemented with 10% heat-inactivated fetal calf serum (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium). PBMC were used for cytoflouorimetric analysis of P2X7 expression.

Flow cytometry measurement of P2X7 expression

PBMC (1×10^6 cells/ml) were incubated overnight in the presence or absence of 3 mM ATP (Sigma) and 100 ng/ml LPS (Sigma), at 37°C/5% CO_2. Cells were washed once in HBSS solution. Cell suspensions (1 ml) were transferred into FACS tubes and labelled with PE-conjugated anti-CD14 (555398; BD Bioscience, USA) and FITC-conjugated anti-P2X7 (P8997; Sigma). PE conjugated IgG1 (555574; BD Bioscience) and Total IgG (R9133; Sigma) were used as isotype controls. Cells were incubated for 30 min at 4°C, washed twice in HBSS solution and resuspended in FACS fixative (4% paraformaldehyde in phosphate buffered saline). Cells were analysed using the Beckman Coulter Epics XL (Fig. 1).

IL-1β and IL-6 ELISA

Peripheral venous blood was collected from each subject in heparin-containing vacuum tubes. Next, 200µl whole blood and 200µl RPMI-1640 medium containing 2 mM L-glutamine (incomplete medium) were transferred into 1.5 ml EP tubes. Diluted blood was incubated in the presence of 100 ng/ml LPS and 3 mM ATP for 2 h at 37°C/5% CO_2. Tubes were centrifuged at 1000×g for 5 min, and the supernatants transferred into freezing tubes and stored at -20°C. The remaining whole blood was centrifuged at 1000×g for 5 min, and the plasma also transferred to freezing tubes and stored at -20°C. IL-1β and IL-6 levels in blood plasma and supernatant after ATP stimulation were measured by enzyme-linked immunosorbent assay (ELISA), using human IL-1β and IL-6 ELISA kits (Multi-sciences, China), according to the manufacturer's instructions.

Statistical analysis

Normally distributed data are presented as mean ± SD and were analysed by Student paired *t*-tests or one-way ANOVAs with LSD tests. Non-normally distributed data are presented as interquartile ranges, and were analysed by Mann-Whitney U or Kruskal-Wallis tests. Two parameters used Pearson or Spearman rank correlation tests. A value of $p<0.05$ was considered statistically significant.

Results

Gender and age differences within the four groups were not statistically different ($p>0.05$). In the pSS group, 45.2% (14/31) patients had anxiety and 32.3 % (10/31) depression.

Surface expression of P2X7 receptors

Surface expression of P2X7R recep-

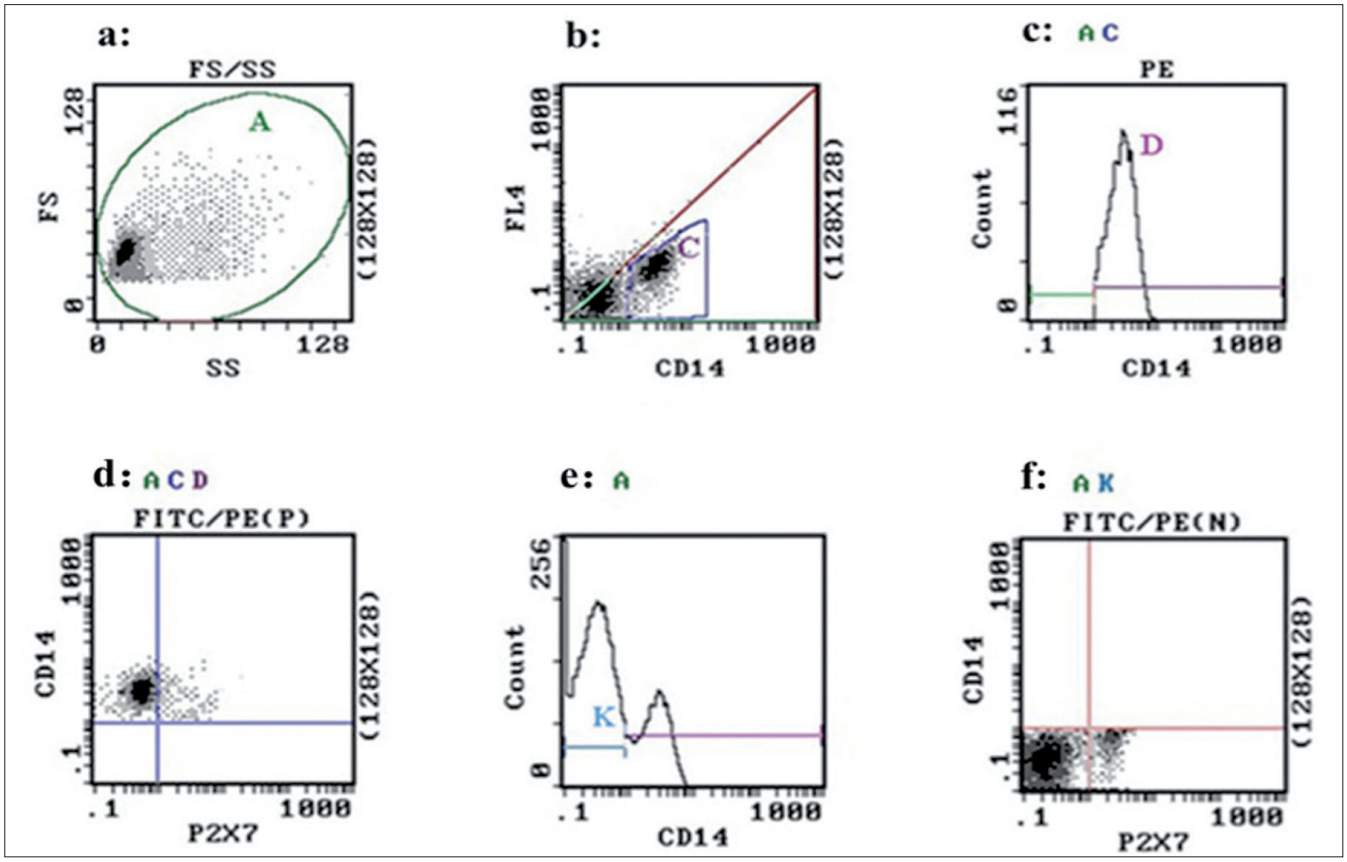

Fig. 1. FCS protocol.
a. In FS/SS pattern, select PBMC into gate A; **b**. By FL2 Channel and FL4 Channel, exclude dead cells; Select FL2 Channel PE fluorescence CD14 positive cells into gate C; **c**. D is $CD14^+$ cells in gate AC; **d**. Count $CD14^+$ P2X7+PBMC in ACD; **e**. K is $CD14^+$ cells in gate A; **f**. Count $CD14^-$ P2X7+PBMC in AK;

tors on PBMC were measured by flow cytometry. P2X7R expression was detected in all subjects. Compared with no ATP stimulation, significant up-regulation of P2X7R expression on $CD14^+$ PBMC (monocytes) was observed after ATP stimulation in the pSS group only (5.63±2.48% *vs*. 3.98±1.68%; t=3.647, p=0.001), while on $CD14^-$ PBMC (predominantly lymphocytes), significant up-regulation was observed in both the pSS (16.92±9.25% *vs*. 10.06±4.35%; t=4.908, p<0.001) and anxiety/depression (12.68±8.15% *vs*.8.07±5.05%; t=3.412, p=0.003) groups. After ATP stimulation, P2X7R expression on $CD14^+$ PBMC in the pSS group was significantly higher than the RA group (5.63±2.48% *vs*. 4.15±2.20%; p=0.044), anxiety/depression group (5.63±2.48% *vs*. 3.35±1.80%; p=0.004) and healthy controls (5.63±2.48% *vs*. 3.25±2.05%; p=0.002). Similarly, P2X7R expression on $CD14^-$ PBMC in the pSS group was significantly higher than the RA group (16.92±9.25% *vs*. 11.40±8.71%; p=0.039) and healthy controls (16.92±9.25% *vs*. 6.51±3.98%; p<0.001), but not the anxiety/depression group (16.92±9.25% *vs*. 12.68±8.15%; p=0.126). Regardless of ATP stimulation, P2X7R expression on $CD14^-$ PBMC from pSS patients with anxiety and depression was significantly higher than those without anxiety or depression (p_{ATP}=0.003; $p_{NO\ ATP}$=0.009) (Table II).

IL-1β and IL-6 levels

IL-1β and IL-6 levels were measured by ELISA. Minimal IL-1β was observed in blood plasma (data not shown). After ATP stimulation, supernatant IL-1β levels were detected in all subjects. In the pSS group, supernatant IL-1β levels were significantly higher than the RA group (18.39±13.99% *vs*. 9.23±6.25%; Z=-2.560, p=0.010), anxiety/depression group (18.39±13.99% *vs*.9.44±5.56%; Z=-2.109, p=0.035) and healthy controls (18.39±13.99% *vs*. 9.93±6.77%; Z=-2.244, p=0.025).

IL-6 levels were detected in all subjects in both blood plasma and supernatant. In healthy controls without ATP stimulation, blood plasma IL-6 levels were significantly lower than the pSS (Z=-2.521, p=0.012), RA (Z=-3.724, p<0.001) and anxiety/depression (Z=-2.406, p=0.016) groups. In the RA group, IL-6 levels were significantly higher than the pSS (Z=-2.647, p=0.008) and anxiety/depression (Z=-2.347, p=0.019) groups. Supernatant IL-6 levels after ATP stimulation were significantly higher than blood plasma levels without ATP stimulation in all groups (Z=-6.793, p<0.001). There were no significant differences in supernatant IL-6 levels in all subjects after ATP stimulation ($\chi 2$=0.778, p=0.855) (Table III).

Correlation analysis

After ATP stimulation, P2X7R expression on $CD14^-$ PBMC was significantly positively correlated to scores of anxiety (r=0.344, p=0.030) and de-

Table II. Cytofluorimetric analysis of P2X7R expression on PBMC (%) (mean ± SD).

GROUP	ATP		NO ATP		*p* (a-c)	*p* (b-d)
	$CD14^+ P2X7^+$ (a)	$CD14^- P2X7^+$ (b)	$CD14^+ P2X7^+$ (c)	$CD14^- P2X7^+$ (d)		
pSS	5.63 ± 2.48[(a)]	16.92 ± 9.25[(b)]	3.98 ± 1.68	10.06 ± 4.35	0.001**	<0.001**
RA	4.15 ± 2.20	11.40 ± 8.71	3.44 ± 2.23	8.76 ± 5.16	0.118	0.079
Anxiety/Depression	3.35 ± 1.80	12.68 ± 8.15	2.39 ± 0.85	8.07 ± 5.05	0.078	0.003**
Normal	3.25 ± 2.05	6.51 ± 3.98	3.47 ± 1.59	7.12 ± 4.02	0.674	0.581
F	5.016	5.157	2.581	1.394		
P[(c)]	0.003**	0.003**	0.059	0.250		

$p<0.05^*$, $p<0.01^{**}$

[(a)] LSD test. After ATP stimulation, P2X7R expression on $CD14^+$ PBMC in the pSS group was significantly higher than the RA group (p=0.044), anxiety/depression group (p=0.004) and healthy controls (p=0.002).

[(b)] After ATP stimulation, P2X7R expression on $CD14^-$ PBMC in the pSS group was significantly higher than the RA group (p=0.039) and healthy controls (p<0.001), but not the anxiety/depression group (p=0.126).

[(c)] One-way ANOVA.

pression (r=0.319, p=0.045). Without ATP stimulation, P2X7R expression was only significantly positively correlated to scores of anxiety (r=0.442, p=0.045) (Table IV). After two hours of LPS and ATP stimulation, supernatant IL-1β levels in the pSS group were significantly positively correlated to P2X7R expression on $CD14^+$ PBMC (r=0.447, p=0.025). There was no significant correlation between P2X7R expression on PBMC, and IL-6, ESR, CRP or immunoglobulins, in the pSS group.

Discussion

In 1988, Angelopoulos (1) investigated personality and psychopathology in patients with primary Sjögren's syndrome, and observed that patients manifested high levels of hostility, as well as anxiety and depression symptoms. Later, smaller studies (2-4) showed that psychiatric disorders, including anxiety and depression, are common in pSS patients, and requires appropriate therapy. Recently Barbara's (5) large investigation showed similar results to these earlier studies. We used the HAMA and HRSD to assess anxiety and depression symptoms in pSS patients, and obtained similar results.

The P2X7 receptor is mainly expressed on macrophages/monocytes, microglia and certain lymphocytes. Many studies show that P2X7R may be related to the pathogenesis of autoimmune disease and psychiatric symptoms through multiple mechanisms. Unlike other P2X receptors, successive studies have demonstrated that P2X7R is distinct, as its downstream signaling is coupled to pro-inflammatory cascades (7, 8). By regulating the release of pro-inflammatory cytokines, P2X7R activity may be involved in immune dysfunction pathophysiology. Inflammatory stimuli (in particular LPS) excite Mθ to produce pro-caspase-1 (Interleukin converting enzyme, ICE) and pro-IL-1β. Extracellular ATP then stimulates P2X7R expressed on Mθ, causing a reduction in intracellular K+ concentrations, and in turn converting pro-caspase-1 to caspase-1. Activated caspase-1 converts inactive pro-IL-β into mature and active IL-1β, which is then released into the extracellular space (7). Pharmacological research (7) has shown that following ATP stimulation, *in vitro* LPS-primed Mθ result in increased maturation and release of IL-1β. This can be reversed in the presence of organic anion-transporting polypeptide (OATP), one of the most widely used P2X7R antagonists. Solle *et al*., (15).have generated a P2X7R-deficient mouse line, showing that absence of P2X7R results in macrophages

Table III. ELISA of IL-1β (pg/ml) and IL-6 (pg/ml) levels (mean ± SD; interquartile range).

GROUP	No LPS/ATP		LPS/ATP 2h	
	IL-1β	IL-6	IL-1β	IL-6
pSS	NA[(a)]	2.12~9.54	18.39±13.99[(b)]	16.65~250.23
RA	NA	5.24~63.92	9.23±6.25	63.40~183.11
Anxiety/Depression	NA	2.39~9.33	9.44±5.56	59.32~131.39
Normal	NA	0.90~3.16	9.93±6.77	12.55~329.79
χ^2	–	18.402	9.716	0.778
P [(c)]	–	<0.001**	0.021*	0.855

$p<0.05^*$, $p<0.01^{**}$

[(a)] No IL-1β was detected.

[(b)] After LPS/ATP stimulation, supernatant IL-1β levels in the pSS group were significantly higher than in the RA group (p=0.010), anxiety/depression group (p=0.035) and healthy controls (p=0.025).

[(c)] Kruskal-Wallis test.

Table IV. Correlation analysis between P2X7R expression on $CD14^-$ PBMC, and anxiety/depression scores.

CD14-P2X7+	Anxiety scores		Depression scores	
	ATP stimulation	NoATP	ATP stimulation	NoATP
r	0.344*	0.442**	0.319*	0.293
P	0.030	0.004	0.045	0.066

$p<0.05^*$, $p<0.01^{**}$.

that are unable to release IL-1β in response to ATP. Moreover, P2X7R can also regulate IL-6 release. In certain chronic inflammatory diseases, such as RA, P2X7R have been implicated in secretion of the pro-inflammatory cytokine, IL-6, from fibroblasts (16). In addition, ATP-stimulation of P2X7R on murine mast cells increases expression of several pro-inflammatory cytokines, including IL-6 (17).

In this study, we have shown that compared with no ATP stimulation, significant up-regulation of P2X7R expression on $CD14^+$ PBMC (mainly monocytes) was observed after ATP stimulation in pSS patients only. After ATP stimulation, P2X7R expression on $CD14^+$ PBMC from pSS patients was significantly higher than controls. This suggests that monocytes from pSS patients are more responsive to ATP stimulation than RA or anxiety/depression patients, or healthy individuals. In the presence of LPS, supernatant IL-1β and IL-6 levels after ATP stimulation were significantly higher than blood plasma levels without ATP stimulation, in all groups. This indicates that purinergic receptors, particularly P2 receptors, may be related to the release of pro-inflammatory mediators such as IL-1β and IL-6, from PBMC. Furthermore, after ATP stimulation, P2X7R expression on $CD14^+$ PBMC in the pSS group was significantly positively correlated to supernatant IL-1β levels, further indicating that P2X7R expression in monocytes may play a role in IL-1β maturation and release. Overall, these results show that following ATP stimulation in pSS patients, not only is P2X7R expression up-regulated in monocytes, but its function is also enhanced.

In addition to an association with immune disorders, P2X7R are also associated with psychological disorders. The P2X7R is considered a critical communication link between the nervous and immune systems (9), and may be involved in the pathophysiology of psychiatric disorders, such as anxiety and depression. Basso *et al*., (18) found that P2X7R KO mice exhibit antidepressant-like profiles in the tail suspension and forced swim tests. Boucher's study (19) also showed similar results. There is considerable evidence that genetic factors play an important role in the pathophysiology of affective disorders, including depressive and anxiety disorders. The *P2X7R* gene (encoding the P2X7 purinergic receptor) is located on chromosome 12q24.31, a locus important for anxiety and depressive disorders, and suggesting a genetic overlap in these group of affective disorders (20). A non-synonymous single nucleotide polymorphism, rs2230912 (P2RX7-E13A, G allele), was shown to be associated with bipolar disorder and unipolar depression by fine mapping and linkage studies (21). Significant association was found between the G-allele of the Gln460Arg polymorphism of the *P2X7R* gene and depressive disorder (22).

P2X7R activity may be involved in development of psychiatric disorders by regulating cytokine release. According to some studies, dysregulation of cytokine levels is common in psychiatric disorders like anxiety and depression. Song *et al*., (23) confirmed that blood IL-1β levels are increased in depressed patients, and can be reduced with treatment. Boufidou *et al*., (24) found that cerebrospinal fluid IL-6 levels are positively associated with depressive mood during postpartum depression. Many animal experiments have also corroborated this finding. Pro-inflammatory cytokines IL-1β and IL-6, cause animals to exhibit obvious depression-like behaviours. Koo *et al*., (25) demonstrated that IL-1RI null mice exhibit behavioural phenotypes consistent with decreased anxiety-related behaviours. Chourbaji *et al*., (26) found that IL-6 (-/-) mice show reduced despair, resistance to helplessness, and enhanced hedonistic behaviour. Moreover, *in vitro* studies show that when incubated with LPS, glial cells cultured from brains of P2X7R(-/-) mice produce less IL-1β, while *in vivo*, IL-1β mRNA is reduced in brains of P2X7R(-/-) mice in response to systemic LPS (24).

Our results show that blood plasma IL-6 levels in pSS and anxiety/depression patients are significantly higher than healthy individuals. Supernatant IL-6 levels after LPS/ATP stimulation are significantly higher than blood plasma levels without stimulation. Compared with no ATP stimulation, significant up-regulation of P2X7R expression in $CD14^-$ PBMC (mostly lymphocytes) was observed after ATP stimulation in both pSS and anxiety/depression patients. After ATP stimulation, P2X7R expression in $CD14^-$ PBMC from pSS patients was significantly higher than RA and healthy individuals, but not significantly different to anxiety/depression patients. Regardless of ATP stimulation, P2X7R expression in $CD14^-$ PBMC from pSS patients with anxiety and depression was significantly higher than those without. After ATP stimulation, P2X7R expression in $CD14^-$ PBMC was significantly positively correlated to scores of anxiety and depression. These results further suggest that $CD14^-$ PBMC from pSS patients are more responsive to ATP stimulation. By regulating the release of pro-inflammatory cytokines and gene polymorphisms, P2X7R activity may be involved in pSS pathogenesis, and in patients with anxiety and/or depression. Therefore, to explore the relationship between pSS and anxiety/depression, we may investigate P2X7R expression in response to ATP, in microglial cells from animal brains. Alternatively, we may compare the G-allele of the Gln460Arg polymorphism of the *P2X7R* gene between pSS patients and those with anxiety and/or depression. As mentioned earlier, studies have implied that P2X7R may play an important role in SS pathogenesis, with salivary glands being the main research organ of these studies. Baldini *et al*., found P2X7R expression significantly higher in salivary glands of pSS patients, with its expression coupled to activation of the inflammasome complex and increased IL-18 release. This suggests that in future research, we will be able to detect IL-18 levels in blood plasma and supernatant after ATP stimulation.

In addition, Al-Shukaili *et al*., used similar methods to analyse IL-1β production and P2X7 expression after ATP stimulation in PBMC from RA patients, and found no difference compared with controls, in ATP-induced P2X7R up-regulation, similar to our results. However, the results of this study show there

is an increased IL-1β response, contrary to our research. There are two explanations for this discrepancy: a) in the early stage (<2h) of ATP-induced P2X7R expression, there is an increased IL-1β response, which has not been detected by us; b) our sample size is too small to detect the difference (27).

In conclusion, psychiatric disorders such as anxiety and depression, are common in pSS patients. Immunological dysregulation is often present in both pSS and psychiatric disorders. The P2X7R is considered a key role between neuropsychiatric disorders and autoimmune disease. Our results indicate that P2X7R may contribute to the complex pathogenesis of pSS and are involved mechanistically in patients with anxiety and/or depression by regulating pro-inflammatory cytokines, especially IL-1β. Furthermore, P2X7R antagonists may have therapeutic potential, and the specific role of P2X7R should be further investigated.

Finally, our research has two main limitations: i) the sample size is small. We will increase the sample size in our following research to confirm our initial findings; ii) the ANA titer is absent in our questionnaire, therefore we cannot determine the relationship between ANA titer and HAMA/HRSD scores in the pSS group.

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