Association of P2X7R Functional Expression and Gene Polymorphisms with Systemic Lupus Erythematosus

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Abstract

Objective: To investigate P2X purinoceptor 7 (P2X7R) expression changes on peripheral blood mononuclear cell (PBMC) surfaces of systemic lupus erythematosus (SLE) patients as well as their association with serum cytokine levels and patients' clinical features. In addition, the effects of different P2X7R single nucleotide polymorphisms (SNPs) on P2X7R expression and cytokine production were investigated.

Method: Twenty-nine new-onset SLE patients and twenty-eight healthy controls were enrolled, while P2X7R expression levels of lymphocytes, CD4+ cells and CD19+ cells were analyzed by flow cytometry. Serum IL-1β, IL-6 and TNF-α levels were analyzed by ELISA and three P2X7R SNPs (1068G>A, 1096C>G and 1513A>C) were PCR genotyped in 14 SLE patients and 14 healthy controls.

Results: P2X7R surface expression levels of lymphocytes, CD4+ and CD19+ cells from new-onset SLE patients were significantly higher than that of controls and significantly elevated on lymphocytes from patients with concurrent arthritis or leukopenia as well as on CD19+ cells of NP-SLE patients. Moreover, P2X7R expression levels on lymphocytes and CD19+ cells of SLE patients were positively correlated with serum TNF-α/IL-6 and TNF-α level, respectively. SLE patients with the P2X7R 1068GG genotype had significantly higher expression level ratios of TNF-α, IL-6 and IL-1β to P2X7R on lymphocytes compared to patients harboring the 1068GA genotype.

Conclusion: P2X7R, as an important cell surface regulator of several key cytokines, is involved in the pathogenesis of SLE and also associated with organ injuries like arthritis, leukopenia and neuropsychiatric damage. P2X7R 1068GG maybe a SLE susceptibility genotype since it enhances cytokine secretion in SLE patients.

Introduction

Systemic lupus erythematosus (SLE) is an inflammatory autoimmune disease characterized by the production of autoantibodies and damage to various organs. SLE pathogenesis includes aberrant autoantibody production, B- and T-cell abnormalities, irregular lymphocyte apoptosis, and dysregulated cytokine production [1-3]. Although the etiology of SLE is still not completely understood, both environmental and genetic factors have been suggested to contribute to the development of SLE. Among these factors, genetic factors are especially important and changes in multiple genes are associated with SLE development [4-6].

The gene encoding the human purinergic receptor P2X ligand-gated ion channel 7 (P2X7 receptor, P2X7R) is located in the 12q24.31 chromosomal region. Importantly, this locus has been recently identified as a SLE susceptibility locus in Hispanic and European American families [7,8]. The P2X7R gene spans 53 kb and consists of 13 exons gene. Furthermore, the P2X7R gene is highly polymorphic and many single-nucleotide polymorphisms (SNPs) have been identified. The P2X7R protein contains 595 amino acids that include two membrane-spanning domains and a long intracellular C-terminus (about 240 amino acids) region that is essential for its function [9]. Many of the P2X7R gene SNPs alter the amino acid sequence and affect P2X7R expression and functions (Figure 1). For example, extracellular domain His-155 to Tyr polymorphism (489C>T) affects Ca²⁺ influx and ethidium uptake and thereby confers a gain-of- function effect [10]. Moreover, the Arg-307 to Gln polymorphism (946G>A) was shown to be critical for ATP binding and receptor activation [11]. In addition, the intracellular domain Thr-357 to Ser polymorphism (1096C > G) alters the binding of P2X7R to ATP [12]. Whereas, the Ile-568 to Asn polymorphism (1729T>A) regulates P2X7R gene expression and functions by preventing intracellular trafficking and localization to the plasma membrane [13,14]. Furthermore, the Glu-496 to Ala polymorphism (1513A>C) was shown to affect the carboxyterminal tail and lead to a loss of receptor function, thus preventing IL-1β and IL-18 release [15-17]. A trans membrane domain Ala-348 to Thr polymorphism (1068A>G) was found to enhance IL-1β secretion [18]. The alteration of P2X7R gene expression and functions induced by SNPs has been show to be involved in pathogenesis of other autoimmune diseases including rheumatoid arthritis [19,20]. It was discovered in animal experiments that the P2X7R gene contains a SLE susceptibility locus [21], indicating that human P2X7R SNPs could be potentially involved in the development of SLE.

However, previous studies showed that SNPs (1513A>C and 762T>C) in the P2X7R gene were not associated with SLE development [22,23]. Similarly, in our previous studies, we did not identify an association of the above SNPs with SLE development. Rather, we found that the 1068G>A polymorphism was associated with SLE development
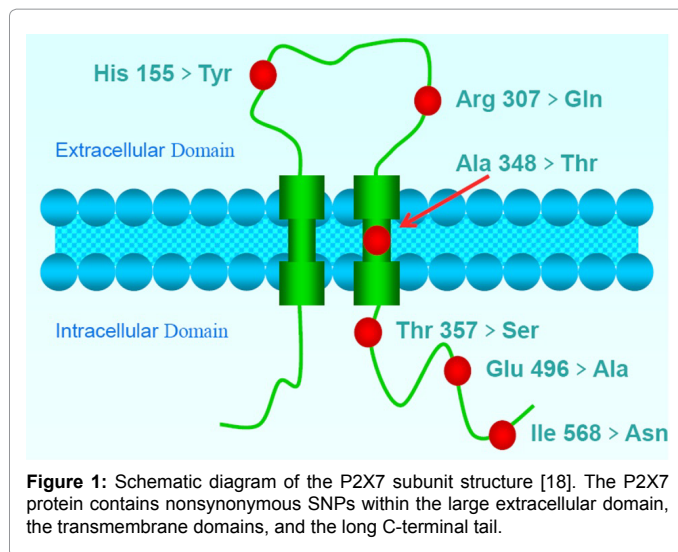
Figure 1: Schematic diagram of the P2X7 subunit structure [18]. The P2X7 protein contains nonsynonymous SNPs within the large extracellular domain, the transmembrane domains, and the long C-terminal tail.

[24], suggesting that dysregulated P2X7R gene expression and function was involved in the development of SLE. The P2X7R gene SNP 1068 G>A changes the alanine to threonine at amino acid 348 and leads to a gain-of-function effect and enhanced IL-1β secretion [18]. It has been hypothesized that alterations in P2X7R expression and function can result in abnormal secretion of inflammatory cytokines including IL-1β, and thus induces the development of SLE. Therefore in the current study we examined the correlation between P2X7R expression levels and serum IL-6, TNF-α, and IL-1β levels in SLE patients and healthy controls. Moreover, we genotyped the three most common loci of P2X7R (1068G>A, 1096C>G and 1513A>C) polymorphisms in the Chinese population. Finally, the association of P2X7R gene polymorphisms and functional expression with SLE development was analyzed.

Materials and Method

Patients and controls

Twenty-nine new-onset untreated SLE patients (female 27, male 2) from the rheumatology facility at Anhui Province Hospital were chosen for this study. The mean age of the patients was 33.83 ± 10.77 years old (range 18–57 years old). All patients fulfilled the 1997 American College of Rheumatology revised criteria for the classification of SLE [25]. Patients who had infections within two weeks, possessed complications from other autoimmune diseases, or had received drug therapy were excluded. A total of 28 randomly selected healthy volunteers (female 26, male 2) were included as the control group. The mean age of the healthy controls was 28.64 ± 8.88 years old (range 18-59 years old). There was no statistically significant difference in the ages between SLE patients and healthy controls (P>0.05). All of the patients and healthy controls gave written informed consent before enrollment, heparinized and non-anticoagulative tube was used to collect peripheral white blood cells from patients and controls. The study protocol was approved by the medical ethics committee of Anhui Provincial Hospital.

Determination of P2X7R expression levels in peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared from the heparinized blood of SLE patients and healthy controls. Cells were stained with 5 ul of APC-CD4 antibody (Clone # RPA-T4, eBioscience, USA), 5 ul of PE-CD19 antibody (Clone # SJ25C1, eBioscience, USA), 10 ul of FITC-P2X7R antibody (Cat #

P8997, Sigma, USA) in the dark at 4 degree for 30 minutes. Cells were washed in twice in PBS, fixed in 200 ul of 1% paraformaldehyde and cellular P2X7R expression levels were determined by flow cytometry.

Cytokine measurement

Two ml of non-anticoagulative blood was collected during the blood drawing from SLE patients and healthy controls. The sera were obtained from non-anticoagulative blood and stored at -80°C. The concentrations of IL-6, IL-1β, and TNF-α were determined by ELISA.

DNA extraction and SNP genotyping

Genomic DNA samples were prepared from PBMCs using the QIAGEN FlexGene DNA kit (Cat.No.51206) according to the manufacturer's recommendations. Three SNPs were genotyped by TaqMan SNP Genotyping Assay Kit using an ABI 7300 Real-Time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA). TaqMan probes used in this study were as follows: C_11704039_10 for rs1718119 (1068G>A), C_15853705-20 for rs2230911 (1096C>G) and C_27495274_10 for rs3751143 (1513A>C). The thermal cycling conditions were as follows: pre-run at 50℃ for 2 min and 95℃ for 10 min; 40 cycles with a 15 sec denaturation step at 95℃, followed by a 60℃ annealing step for 1 min.

Statistical analysis

Data were analyzed by SPSS 10.1 software. χ^2 test was used to compare groups with qualitative data. Quantitative data were presented as mean ± standard. For quantitative data, *t* test was used to compare two groups with normal distribution and Mann-Whitney U rank sum test was used to compare two groups without normal distribution. The correlation analysis for data with and without normal distribution was analyzed by Pearson correlation and Spermann ranking correlation, respectively. $P<0.05$ was considered as statistically significant.

Results

Analysis of surface P2X7R expression levels in the peripheral blood of new-onset SLE patients

Surface P2X7R expression levels on lymphocytes, CD4+ cells, and CD19+ cells in new-onset SLE patients were all significantly higher than that of healthy controls (lymphocytes: 4.42 ± 5.46 vs. 1.31 ± 0.72, CD4+ cells: 3.72 ± 4.60 vs. 1.53 ± 2.03, CD19+ cells: 15.90 ± 12.58 vs. 9.21 ± 7.67) (P<0.05) (Figure 2). The correlation between P2X7R expression levels in different cell populations and Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) was analyzed. The results showed that P2X7R expression on lymphocytes and CD19+ cells was positively correlated with SLEDAI-2K (lymphocytes: r=0.374, P=0.005; CD19+ cells: r=0.274, P=0.041). No significant correlation was identified between P2X7R expression on CD4+ T cells and SLEDAI-2K(r=0.225, P=0.095).

P2X7R expression profiles in SLE patients with different clinical features

SLE patients were divided into different groups based on clinical features. The following clinical symptoms were used to group SLE patients: peripheral joint swelling and pain in 2 or more joints (arthritis group), cheek erythema (erythra group), urine protein index greater than 3 or 24-hour urinary protein excretion greater than 0.5 gram (nephritis group), neuropsychiatric symptoms (NP-SLE group), white blood cell that are less than 4×10^9/L (leukopenia group), and platelets that are less than 10×10^9/L (thrombocytopenia group). SLE patients
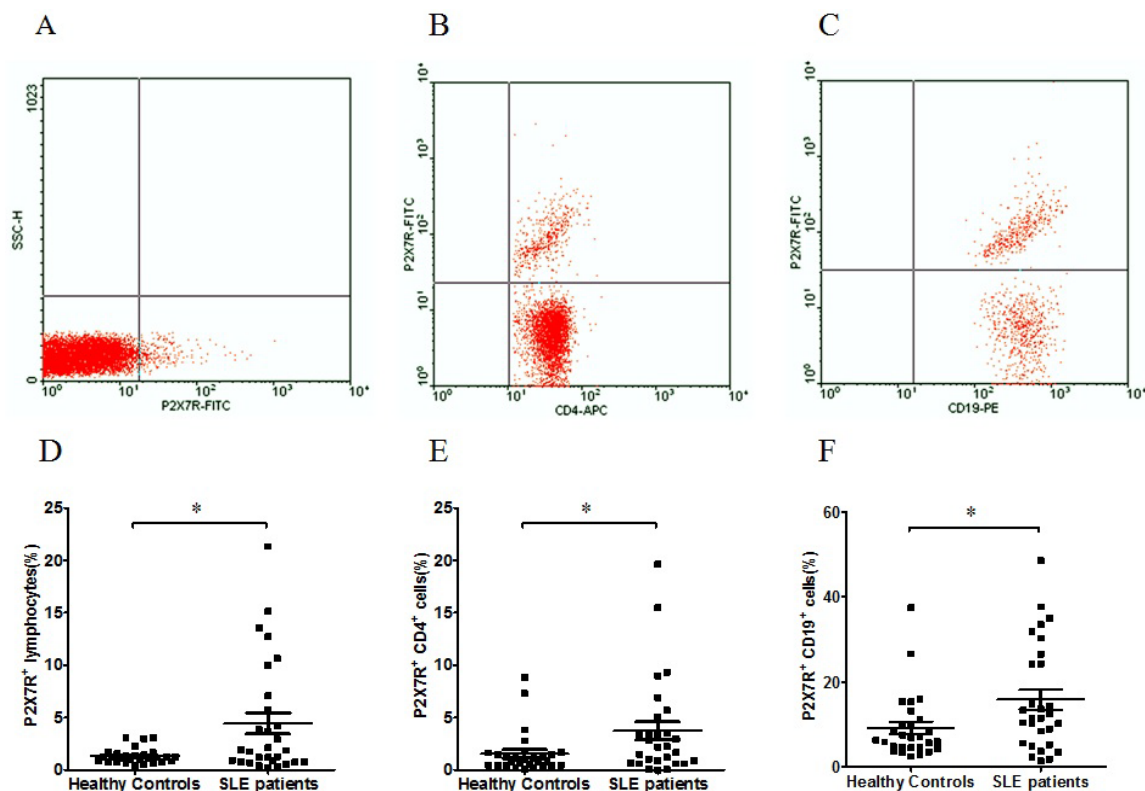
Figure 2: The comparison of PBMC P2X7R expression levels between new-onset SLE patients and healthy controls. A. Representative FACs plots for P2X7R expression on lymphocytes. B. FACs plots for P2X7R expression on CD4+ cells. C FACs plots for P2X7R expression on CD19+ cells. D. Comparison of P2X7R expression on lymphocytes. E. Comparison of P2X7R expression on CD4+ cells. F. Comparison of P2X7R expression on CD19+ cells.

without any of the above clinical features were included in the negative group. The P2X7R expression levels in SLE patients with different clinical features were compared. The results showed that P2X7R expression levels on lymphocytes in the arthritis and leukopenia groups were significantly higher than that of non-arthritis groups or groups with normal leukocyte number. Moreover, P2X7R expression on CD19+ cells in the NP-SLE group was significantly higher than that of non-NP-SLE group ($P<0.05$) (Tables 1 and 2).

The association between P2X7R expression and serum cytokine changes

Considering that cytokine concentrations in SLE patients can be influenced by various factors, we did a separate correlation analysis between all SLE patients and healthy controls. The correlation analysis between P2X7R expression levels and serum cytokines showed that P2X7R expression levels on PBMC lymphocytes and CD19+ cells in SLE patients were positively correlated with serum TNF-α and IL-6 levels. Moreover, P2X7R expression levels on PBMC CD19+ cells in healthy controls were positively correlated with serum IL-1β and IL-6 levels.

P2X7R expression and cytokine levels in patients with different P2X7R gene SNPs

In the above subjects, 14 SLE patients and 14 healthy controls were randomly selected to genotype the 1068G>A, 1513A>C, and 1096C>G SNP loci of the P2X7R gene. In our previous study, we found the following genotype frequencies in SLE patients and healthy controls, respectively: AA genotype frequency in 1068G>A, 0.670 and 3.430%; CC genotype frequency in 1513A>C, 4.160 and 5.025%; GG genotype

frequency in 1096C>G, 3.005 and 2.771%, all of which are very low [24]. In the current study, we did not identify the above genotypes in the chosen subjects. The distributions of the genotypes for the subjects included in this study are provided in Table 3.

Cytokine levels and P2X7R expression were compared between 1068GA and 1068GG, 1513AC and 1513AA, 1096CG and 1096CC in SLE patients. The results showed that SLE patients possessing the 1068GG polymorphism had significant reductions in P2X7R expression on lymphocytes relative to SLE patients harboring the 1068GA polymorphism. No significant differences were identified in P2X7R expression in SLE patients with other genotypes and in cytokine production among all genotypes (Figure 3). In addition, there were no significant differences in P2X7R expression and cytokine levels among healthy controls with different genotypes.

Comparison of capacity to secrete cytokines among SLE patients with different P2X7R SNPs genotypes.

The ratios of serum cytokine levels to P2X7R expression on lymphocytes reflect the capability of P2X7R genotypes to stimulate cytokine production. It was demonstrated that the ratios of TNF-α, IL-6, and IL-1β to P2X7R expression on lymphocytes of SLE patients with the 1068GG polymorphism was significantly higher than that of SLE patients possessing the 1068GA polymorphism, whereas there were no significant differences in these ratios among healthy controls. Regardless of the SLE patients or healthy controls, no significant differences were identified in the ratios of TNF-α, IL-6, and IL-1β to P2X7R expression on lymphocytes between 1513AG and 1513AA or 1096CG and 1096CC (Figure 4).

Clinical Features		n	lymphocytes	CD4+ cells	CD19+ cells
Arthritis	positive	15	6.91 ± 6.50*	3.44 ± 4.11	15.82 ± 10.73
	negative	14	1.75 ± 1.95	4.01 ± 5.22	15.98 ± 14.72
Erythra	positive	18	3.15 ± 3.45	4.09 ± 4.02	16.88 ± 12.65
	negative	11	6.49 ± 7.46	3.11 ± 5.58	13.47 ± 11.54
Nephritis	positive	15	4.41 ± 5.66	4.27 ± 5.10	15.31 ± 12.81
	negative	14	4.46 ± 5.23	1.96 ± 1.94	16.52 ± 12.77
NP-SLE	positive	3	7.65 ± 6.47	3.30 ± 0.26	34.37 ± 3.73*
	negative	26	3.45 ± 4.96	3.65 ± 1.80	13.77 ± 11.51
Leukopenia	positive	16	6.93 ± 6.30*	4.45 ± 5.68	12.62 ± 13.30
	negative	13	1.33 ± 1.11	2.82 ± 2.74	18.56 ± 11.71
Thrombocytopenia	positive	10	5.90 ± 7.58	2.09 ± 1.58	20.21 ± 15.66
	negative	19	3.64 ± 3.98	4.57 ± 5.42	13.63 ± 10.38

* $P<0.05$

Table 1: The frequency comparison of surface P2X7R expression on PBMCs among SLE patients with different clinical features.

Cytokine (pg/ml)		lymphocytes (%)		CD4+ cells (%)		CD19+ cells (%)	
		SLE	control	SLE	control	SLE	control
IL-6	r_s	0.449*	0.077	-0.155	0.369	0.339	0.552**
	P	0.015	0.698	0.422	0.053	0.072	0.002
TNF-α	r_s	0.636**	0.075	0.242	-0.087	0.635**	0.135
	P	0.000	0.705	0.206	0.660	0.000	0.494
IL-1β	r_s	0.104	0.138	0.337	0.070	0.178	0.409*
	P	0.590	0.485	0.074	0.725	0.355	0.030

* $P<0.05$, ** $P<0.01$

Table 2: Correlation analysis between P2X7R expression on different cells and serum cytokines levels.

genotype		SLE	control
1068	GA	3	5
	GG	7	6
1513	AC	5	5
	AA	8	7
1096	CG	4	8
	CC	10	5

Table 3: Distribution of P2X7R gene SNPs genotype.

Discussion

As an inflammatory autoimmune disease, SLE is induced by abnormal immune cells and secretion of inflammatory cytokines (IL-1β, TNF-α, and IL-6), inducing subsequent tissue damage and inflammation [2,26]. Inhibition of inflammatory cytokines is one of the most promising therapeutic strategies to treat SLE [27]. P2X7R is a ligand-gated cationic channel receptor and belongs to the ATP-gated ion channel family (P2X1-7). Unlike other P2X receptors, P2X7R downstream signaling is coupled to pro-inflammatory cascades. Therefore, the P2X7R mainly becomes activated in pathological conditions and plays a critical role in regulating the secretion of pathogenic inflammatory cytokines. P2X7R is activated by ATP and is normally expressed on a variety of immune cells, including macrophages and lymphocytes. Moreover, cellular immune regulation disorder is main cause of SLE, such as lymphocytes, CD4+ and CD19+ cells [28], indicating P2X7R may involved in the pathogenesis of SLE via immunocytes. The transient stimulation of P2X7R by ATP results in an immediate influx of cations (Na^+, K^+, Ca^{2+}), cell degranulation, and then release of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6. Subsequently, a feed-forward inflammatory loop is activated, which potentially leads to tissue damage and SLE development [29,30].

In this study, we demonstrate that P2X7R expression on

lymphocytes, CD4+ cells, and CD19+ cells is significantly higher than that of healthy controls. In addition, P2X7R expression on lymphocytes was positively correlated with TNF-α and IL-6 secretion, as well as SLE development. These results suggest that abnormal P2X7R expression could participate in the development of SLE through the regulation of inflammatory cytokine secretion. However, in contrast to our results, Liliana Portales-Cervantes et al [23] showed that there were no statistically significant differences in P2X7R expression on CD4+ and CD19+ cells between SLE patients and healthy controls. This inconsistency could be due to the different experimental subjects between the studies. The subjects we chose were new-onset and untreated SLE patients, who were less likely to be affected. More importantly, SLE is a heterogeneous disease and its pathogenesis could be different depending on diverse clinical characterizations. Therefore, the discrepancy could be the result of SLE patients with different clinical features that were selected in these studies. We found that P2X7R expression was mainly elevated in SLE patients with arthritis, leukopenia and NP-SLE, indicating that P2X7R plays a more important role in inducing tissue damage and SLE development in these patients.

Activation of P2X7R and subsequent abnormal cytokine secretion is one of the most important pathogenic mechanisms in the development of arthritis. It was shown that human rheumatoid synoviocytes express functional P2X7R and BzATP (P2X7R agonists) could induce an increase in IL-6 gene expression and protein production. [31]. In addition, P2X7-deficient mice exhibit resistance to antibody-induced arthritis and impaired IL-1β secretion [32]. In the presence of lipopolysaccharide, ATP can stimulate PBMC from rheumatoid arthritis patients to produce potent amounts of IL-1β [33]. All of the above studies support the hypothesis that abnormal P2X7R expression could induce dysrgulated cytokine production and thus arthritis development. Our results identify that elevated P2X7R expression in SLE patients is associated with arthritis, suggesting that abnormal P2X7R expression in involved in the pathogenesis of SLE.

In contrast to the development of arthritis, P2X7R induces the development of leukopenia possibly through the induction of cell death. Activation of P2X7R by high concentrations of ATP and prolonged exposure to ATP can lead to cell death by different means [34-36]. The mixed apoptotic/necrotic phenotype of P2X7R-stimulated cells is consistent with a potential role for P2X7R in cell death pathways [37]. In fact, cell death-induced leukopenia, which is characterized by loss of lymphocytes, is not only a major manifestation of SLE, but also an important contributor to the pathogenesis of SLE [38-41]. Abnormal P2X7R expression could be an important inducer of cell death. Unfortunately, our study did not examine the effect of P2X7R expression on cell death.

In addition to arthritis and leukopenia, our study also identifies an association of abnormal P2X7R expression with neuropsychiatric damage. Animal experiments have shown that abnormal P2X7R expression can result in nervous system damage. Specifically, P2X7R can affect nerve cell death through the regulation of IL-1β. The activation of P2X7R and subsequent IL-1β release is involved in inflammatory injury in the hippocampus [42]. P2X7R also plays an important role in the development of psychiatric disease. One study found that P2X7R gene polymorphisms are associated with the incidence of depressive disorders [43,44]. Activation of P2X7R can lead to depressive disorders through multiple mechanisms, including stimulating nerve endings to release the neurotransmitter and activating STAT3 to stimulate astrocyte proliferation [45]. P2X7R-associated cytokines such as TNF-α and IL-6 are involved in the incidence of major depressive disorders
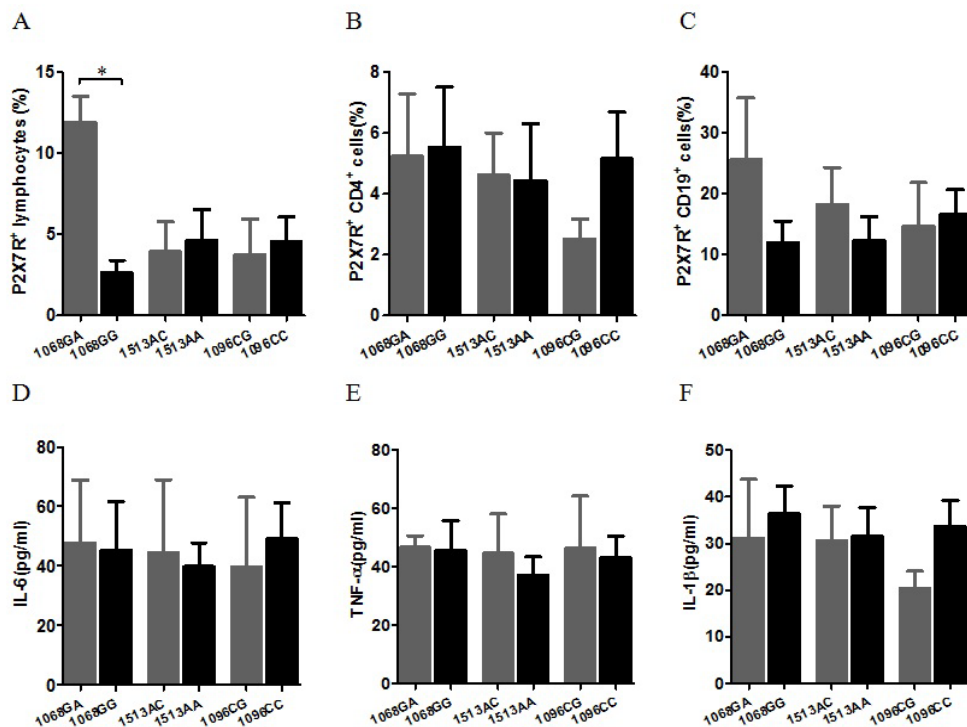
Figure 3: P2X7R expression and cytokine levels in SLE patients with different P2X7R gene SNPs genotypes. As shown in A, there were statistically significant differences in P2X7R expression on lymphocytes between SLE patients with the 1068GA genotype and those with the 1068 GG genotype. No significant differences were identified in P2X7R expression in SLE patients with other genotypes and in cytokine production among all genotypes. * $P<0.05$.
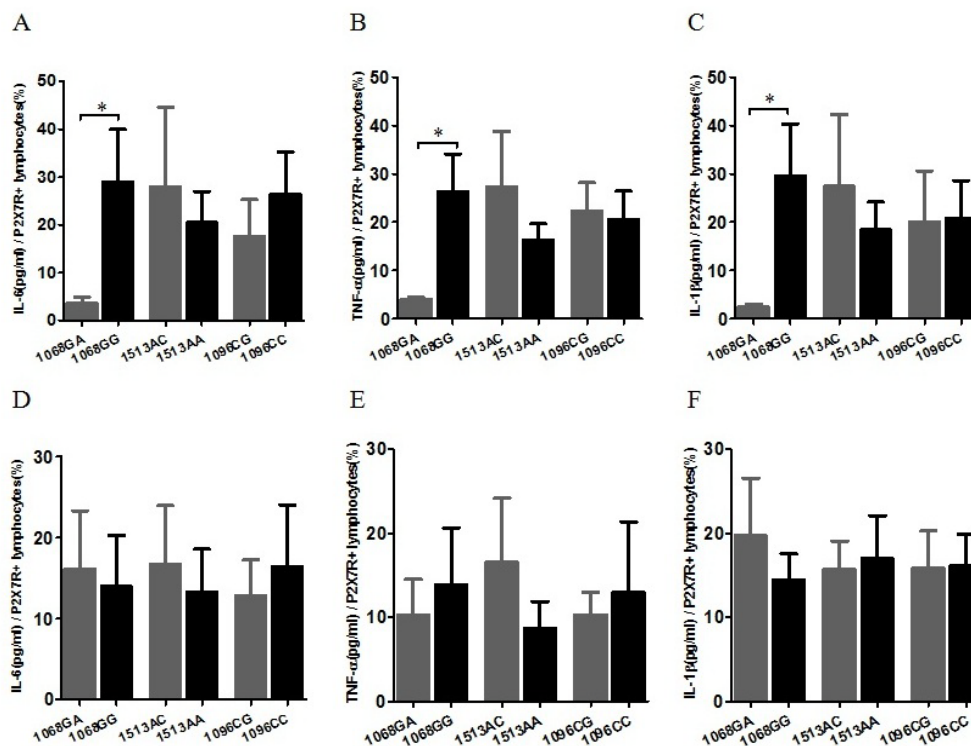

Figure 4: Ratio comparison of cytokine levels to P2X7R expression on lymphocytes in both SLE patients with different P2X7R SNPs genotypes and healthy controls. A, B, C were from SLE patients. The results showed that patients with the 1068GG polymorphism had greater ratios of TNF-α, IL-6, and IL-1β levels to lymphocyte P2X7R expression than patients with 1068GA. D, E, F are were from healthy controls. There were no significant differences in ratios of TNF-α, IL-6, and IL-1β levels to lymphocyte P2X7R expression among all groups. * $P<0.05$.

[46,47]. All of the above studies suggest that P2X7R is involved in the pathogenic development of neuropsychiatric damage. Thus it is not difficult to understand the high levels of P2X7R expression in NP-SLE groups in the current study.

Similar to TNF-α and IL-6 secretion, IL-1β secretion is one of the main functions of P2X7R. However, in the current study, no positive correlation between serum IL-1β and P2X7R expression in SLE patients was identified. In contrast there was a positive correlation in healthy controls. This suggests that alterations in P2X7R function may play a role in SLE pathogenesis. The capability of P2X7R to stimulate IL-1β release can be influenced by P2X7R polymorphisms. Consistent with previous studies [18], SLE patients with the 1068 GG polymorphism had significantly higher ratios of serum TNF-α, IL-6, and IL-1β levels to lymphocyte P2X7R expression than SLE patients with 1068 GA genotype. It suggests that gene SNP 1068 G>A change from alanine to threonine at amino acid 348 of P2X7R markedly increases the ability of P2X7R to release IL-1β.

We also found that SLE patients with the 1068GA polymorphism had dampened P2X7R expression levels and similar concentrations of serum TNF-α, IL-6, and IL-1β compared to those with the 1068GG genotype, even though they had enhanced ability to stimulate IL-1β secretion through P2X7R. So far, no previous studies have found that Ala-348 could affect the expression levels of P2X7R. The reduction of lymphocyte P2X7R expression in SLE patients with the 1068GG genotype can be a result of a negative feedback inhibition by P2X7R-induced cytokine secretion. It is this negative feedback inhibition that potentially results in the similar serum cytokine levels in patients with both the 1068GA and 1068GG genotype. Unfortunately this research also reveals some unavoidable defects, at the same time the sample size for P2X7R expression and gene polymorphism detection is too small. Most importantly, no in vitro test was performed to verify that 1068GG SLE patients have significantly increased ability in P2X7R-induced IL-1β secretion. In addition, there is no literature or experiment support to show if IL-1β conduces to P2X7R expression suppression.

Despite the deficiencies, this research still proves that P2X7R is involved in the pathogenesis of SLE and especially associated with the onset of arthritis, leukopenia and neuropsychiatric damage in SLE patients. Furthermore, the function of P2X7R in the development of SLE could be affected by P2X7R gene polymorphisms. Our previous study showed that the allele frequency of SNP locus 1068G increased in SLE patients. In this study, we also showed that enhanced IL-1β secretion in SLE patients with P2X7R 1068GG genotype in comparison with those with 1068AG. All of these studies suggest that the P2X7R 1068GG is a SLE susceptible genotype. Enhanced secretion of cytokines such as IL-1β by P2X7R 1068 GG genotype may critically contribute to the pathogenesis of SLE.

Acknowledgment

Conflict of Interest

The authors declare that they have no conflict of interest.

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