Increased Spontaneous But Decreased Mitogen-stimulated Expression and Excretion of Interleukin 18 by Mononuclear Cells in Patients with Active Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** To measure serum concentration and analyze the expression of interleukin 18 (IL-18) mRNA in mononuclear cells of patients with systemic lupus erythematosus (SLE).

Methods. IL-18 concentrations in sera and culture supernatants of peripheral blood mononuclear cells (PBMC) from healthy controls and patients with active SLE were measured by ELISA. PBMC and polymorphonuclear leukocytes (PMN) purified from patients with active SLE were stimulated with phytohemagglutinin (PHA), pokeweed mitogen (PWM), and lipopolysaccharide (LPS). Expression of IL-18 mRNA in cells was analyzed by RT-PCR.

Results. Serum IL-18 levels were significantly higher in SLE patients than in controls, and correlated with disease activity in SLE patients (r^2 = 0.602). Two patients receiving intravenous methylprednisolone therapy (1.0 g/day for 3 days) showed profound decreases in serum IL-18 levels after therapy. The quiescent PBMC from SLE patients (30/30) expressed IL-18 transcript more frequently than control PBMC (20/30). PBMC from SLE patients produced more IL-18 than control PBMC after 72 hours of incubation, by RT-PCR. PHA and PWM inhibited the production of IL-18 in PBMC from both SLE patients and controls. Inhibition by PWM was more pronounced than that by PHA, especially in SLE-PBMC. Control and SLE-PMN with or without LPS stimulation produced negligible IL-18.

Conclusion. IL-18 is involved in the autoimmune derangement of leukocyte function in patients with active SLE. (J Rheumatol First Release July 15 2009; doi:10.3899/jrheum.081197)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS INTERLEUKIN 18 MONONUCLEAR CELLS POLYMORPHONUCLEAR LEUKOCYTES

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by imbalance between the production of T-helper 1 cell (Th1) and T-helper 2 cell (Th2) cytokines. Interleukin 18 (IL-18) is a cytokine produced by antigen-presenting cells including macrophages and dendritic cells, which act synergistically with IL-12 on the Th1 cells and natural killer (NK) cells[1-5]. Investigations have shown that IL-18 production is elevated in patients with adult onset Still's disease[6,7], crescentic glomerulonephritis[8], rheumatoid arthritis[9], Crohn's disease[10], and biliary cirrhosis[11]. Several investigators have also reported high serum concentrations of IL-18 in patients with systemic autoimmune diseases including SLE, and concentration was correlated with disease activity[12-16]. Disregulated functions of leukocytes, including neutropenia, lymphocyte dysfunction, and increased susceptibility to opportunistic infections, have been observed in patients with SLE. Whether IL-18 plays a synergistic or an antagonistic role in the leukocyte dysfunction in SLE remains largely unknown. We described a paradoxical decrease in IL-12 production by interferon-γ-primed neutrophils in patients with active SLE[17]. We have demonstrated that this defect did not originate from the lack of IL-12 gene in neutrophils of lupus patients. Since IL-18 acts in concert with IL-12 on T cell and NK cell functions[2], it would be of interest to know whether IL-18 expression and production in SLE patients is also abnormal.

In this investigation, we measured the serum concentration of IL-18 and IL-18 expression by mononuclear cells (including antigen-presenting cells and lymphoid cells) as

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well as neutrophils in patients with active SLE. We found that in resting status, the PBMC had a higher capacity to express IL-18 in SLE patients. When PBMC were stimulated by phytohemagglutinin (PHA) or pokeweed mitogen (PWM), these cells exhibited a decrease of IL-18 expression in both SLE patients and healthy controls. In particular, PWM showed a more potent inhibitory effect on IL-18 gene expression and protein production in SLE-PBMC. On the other hand, neutrophils stimulated by lipopolysaccharide (LPS) seemed not to produce more IL-18. The serum level of IL-18 was higher in patients with active SLE and may be decreased after high-dose steroid pulse therapy.

MATERIALS AND METHODS

SLE patients and controls. Thirty patients (27 women, 3 men) fulfilling 4 or more of the 1982 and 1997 updated American College of Rheumatology criteria for the classification of SLE[18,19] were enrolled for study. The disease activity of SLE was evaluated by serologic and hematologic findings and the SLE Disease Activity Index (SLEDAI) score system[20]. A spot-urine sample was collected from all patients with or without renal involvement for measurement of urine protein-urine creatinine ratio, which was used as a surrogate marker for daily urine protein excretion[21]. The serologic tests used were serum levels of complements (C3 and C4), anti-dsDNA antibodies, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). Thirty age-matched healthy individuals (16 women, 14 men) who came to this hospital for routine physical examinations served as controls.

All patients and controls provided informed consent, and the experiment procedures were approved by the Institutional Review Board in this hospital (VGHIRB95-08-20A).

Isolation of PBMC and PMN from peripheral venous blood. Heparinized venous blood from patients and controls was mixed with one-quarter volume of 2% dextran (500 kDa) and incubated at room temperature for 30 min. Leukocyte-enriched supernatant was collected and diluted with the same volume of Hanks' balanced salt solution cushioned on Ficoll-Hypaque (specific gravity 1.077) density gradient. After centrifugation at 300 g for 25 min, the PBMC were collected from the interface and the PMN were obtained from the bottom. The residual erythrocytes in PMN were disrupted by incubation of cells for 10 min with 0.83% chilled ammonium chloride solution. Purity and viability of the PMN and PBMC were both greater than 95% as confirmed by Wright's stain and trypan blue dye exclusion, respectively. Less than 1% of monocytes contaminated the PMN preparation as detected by nonspecific esterase stain kit (Sigma-Aldrich, St. Louis, MO, USA).

Cultures of the PBMC and PMN. 500 ml of PBMC (2×10^6/ml) or PMN (1×10^7/ml), 0.1 ml (10 μg/ml) of PHA (in the case of PBMC), PWM (in the case of PBMC) or LPS 1 μg/ml (in PBMC and PMN), and 0.4 ml of 10% fetal bovine serum in RPMI-1640 medium (FBS-RPMI) were mixed and incubated at 37°C in 5% CO_2 incubator. In medium-control experiments, PHA, PWM, or LPS was replaced by the same volume of RPMI-1640. For study of IL-18 expression, the cells were cultured for 24 h before extraction of the total cell RNA, and for the IL-18 excretion study, the cells were cultured 72 h before harvest of the supernatants.

Quantification of IL-18 concentration in sera and culture supernatants. PBMC culture supernatants and sera obtained from SLE patients and controls were collected for quantification. The commercial ELISA kits for IL-18 were purchased from R&D Systems, Minneapolis, MN, USA. Detailed assay procedures were carried out as described by the manufacturer. The detection limit of the kit was 25.6 ~ 1000 pg/ml.

Detection of IL-18 mRNA expression in SLE and control PBMC or PMN by reverse transcription-polymerase chain reaction (RT-PCR). PBMC and PMN were cultured with or without mitogen/LPS stimulation for 24 h. At the end of culture, cells were harvested for purification of total cell RNA and RT-PCR. Purification of RNA and RT-PCR was carried out as described, with some modifications[22-24]. Briefly, cultured PBMC (5×10^6 cells) or PMN (1×10^7 cells) were collected. After washes with 0.15 M ice-cold phosphate buffered saline, pH 7.2, total cell RNA was extracted using an RNA purification kit (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The cell pellets were disrupted by lysing buffer. After homogenization with a 20-G needle syringe, the cell lysate was subjected to purification and RNA was purified accordingly. After a wash with buffers, the RNA was dissolved in 30 μl of RNase-free water. The OD_{260}/OD_{280} ratio of the absorbance was kept between 1.8 and 2.1 for all RNA preparations. The amount of RNA was determined by spectrophotometry.

Five micrograms of purified RNA was denatured at 70°C for 5 min and chilled in an ice-bath. The cDNA was generated by reverse transcription of denatured RNA after reaction at 42°C for 60 min in a final volume of 25 μl with reaction solution containing 5 μl of 5× buffer (Promega, Madison, WI, USA), 1.5 μl of 100 mM dithiothreitol (BRL), 1.5 μl of dNTP (dATP, dCTP, dGTP, dTTP, 10 mM each; Promega), 1 μl of 1 mM random hexamer primers (oligo-dT; Promega), 1 μl of Moloney murine leukemia virus (M-MLV)-derived reverse transcriptase (200 U/μl; Promega), and appropriate volume of RNase-free water. Samples were heated 5 min at 75°C and chilled on ice for PCR.

Forty-five microliters of PCR mixture were added to 5 μl of cDNA product. The PCR mixture contained 5 μl of 10× buffer (Finnzymes; Oy Riintontuntie, Espoo, Finland), 2 μl of dNTP, 35 μl of sterile water, 1 μl of 20 μM forward primers and 1 μl of 20 μM reverse primers of IL-18[25] and 1 μl of DNA polymerase (2 U/μl; Finnzymes). The reaction mixture was amplified using a HYBAID OmniGene DNA Thermal Cycler (Teddington, Middlesex, UK) for 35 cycles with a program of denaturing at 94°C for 45 s, annealing at 60°C for 45 s, and primer extension at 72°C for 120 s. The reaction was stopped with a final extension at 72°C for 7 min, followed by incubation at 4°C. The DNA fragment amplified by the primers was 527 bp for IL-18. A 452-bp fragment of ubiquitously expressed GAPDH (Clontech Laboratories) was used as the internal control in the whole amplification procedure. PCR products were analyzed with 1.6% agarose gel electrophoresis using a 100-bp DNA ladder (Bioman Scientific, Taiwan) as the calibration marker. The forward and reverse primers for IL-18 were as follows: human IL-18: 5'ACC TGC TGC AGT CTA CAC AG3' (forward); and 5' GTC CTG GGA CAC TTC TCT GA3' (reverse).

The amount of the PCR product was estimated by ImageMaster TotalLab (Amersham Pharmacia Biotech, Hong Kong).

Statistical analyses. Data are represented as range (median) or mean ± standard deviation. Statistical analyses were carried out with SPSS for Windows software (Release 15.0). p values < 0.05 were regarded as statistically significant.

RESULTS

Patients with SLE. As shown in Table 1, patients with SLE were all in the active stage of the disease (SLEDAI score 9~49, median 26). Two patients (Patients 5 and 6) received large doses of intravenous methylprednisolone infusion (1000 mg daily for 3 days) during the period when samples were collected and experiments were carried out. These 2 patients' peripheral venous blood samples were collected 1 week after therapy for the additional quantification of IL-18 by ELISA.

Serum level of IL-18 in patients with active SLE. The IL-18 concentration in serum was detected with ELISA (R&D Systems). The average concentration of IL-18 in SLE patients was significantly higher than that of controls

Table 1. Demographic characteristics of patients with active SLE.

Patient	Sex	Age, yrs	C3/C4, mg/dl	ESR/CRP, mm-h/mg-dl	Anti-dsDNA, IU/ml	Urinary Protein/ Creatinine Ratio	SLEDAI Score	Remarks
1	F	19	31.4/4.13	70/0.42	585	0	23	
2	F	64	105/26	24/< 0.31	22	0	17	
3	F	37	85.6/17.9	30/< 0.31	21	0.22	36	
4	F	37	79.4/12.0	10/< 0.31	242	0	22	
5	F	35	73.7/17.1	87/2.31	0	0	26	IV methylprednisolone, transverse myelitis
6	F	26	68.3/14.5	58/< 0.31	69	2.89	28	IV methylprednisolone
7	F	40	92.6/15.1	25/2.54	236	1.70	33	
8	F	33	51.8/12.0	93/< 0.31	362	7.66	41	Under cyclosporine A
9	F	53	50.3/9.24	28/1.04	47	2.51	19	
10	M	44	86.3/18.3	6/< 0.31	244	0.83	17	Pleurisy, fibrothorax
11	F	14	68.1/15.3	50/< 0.31	212	2.57	30	
12	F	15	98.6/18.8	8/< 0.31	9	0.38	28	
13	F	50	65.5/15.0	24/< 0.31	28	1.32	12	
14	F	29	83.5/7.27	72/< 0.31	116	12.8	49	Optic neuritis
15	F	23	48.6/10.3	59/1.15	211	4.85	44	
16	F	19	71.6/5.64	8/< 0.31	30	0.38	19	Cutaneous LE
17	F	29	74.2/5.47	50/< 0.31	255	1.19	22	
18	F	56	100/21.9	130/0.98	45	0.93	38	
19	F	26	88.1/17.5	37/< 0.31	50	0	14	CNS lupus
20	F	58	147/34.7	45/< 0.31	6	2.10	35	
21	F	54	100.7/24.5	28/3.59	48	0	9	Overlap with scleroderma
22	F	29	30.4/1.76	7/< 0.31	104	0	13	Evans syndrome
23	F	72	53.7/12.3	1306/7.56	584	0.56	42	Hydronephrosis, recurrent UTI & strokes
24	M	24	37.0/5.21	99/< 0.31	336	2.92	39	CNS lupus
25	M	25	92.8/9.65	35/< 0.31	43	1.14	21	
26	F	31	72.3/11.6	43/< 0.31	181	0.87	38	
27	F	66	105/24.2	55/< 0.31	17	0.53	26	Vasculitis with leg ulcer
28	F	25	82.4/19.9	64/< 0.31	322	3.99	28	
29	F	50	54.9/13.8	51/11.7	78	0.60	21	
30	F	26	74.6/16.9	121/4.96	279	0.86	45	Recurrent strokes

SLEDAI: SLE Disease Activity Index; IV: intravenous; LE: lupus erythematosus; CNS: central nervous system; UTI: urinary tract infection.

(103.82 ± 58.05 pg/ml in SLE vs 54.75 ± 42.62 pg/ml in controls; $p < 0.001$, Mann-Whitney U test). The patients (Patients 5 and 6) undergoing high-dose methylprednisolone therapy showed marked decrease in serum level of IL-18 (Figure 1) after the therapy, suggesting that high IL-18 level may be associated with high activity of autoimmune processes.

Inhibition of PBMC production of IL-18 by PHA and PWM in patients with active SLE. The capacity of IL-18 production in PBMC (500 μl of 2×10^6/ml cells) of patients with SLE was measured by ELISA (R&D Systems). The isolated PBMC were stimulated with various mitogens for 3 days and the culture supernatants were measured for the concentration of IL-18. As shown in Figure 2, the baseline and LPS-stimulated IL-18 production was high. However, IL-18 production was markedly suppressed in the presence of PHA or PWM, especially in the presence of the latter. Production of IL-18 in the normal PBMC was also measured and a similar tendency was also noted (data not shown). These results were consistent with those of the transcript measurement presented below.

Correlation of serum IL-18 level to disease activity in patients with active SLE. All patients' clinical and laboratory characteristics were evaluated at the time of blood sample collection. SLEDAI scores of these patients were correlated to the serum IL-18 concentrations ($r^2 = 0.602$, $p < 0.01$, calculated by linear regression).

IL-18 expression in mitogen- or LPS-stimulated PBMC or PMN. Baseline and PHA-, PWM-, or LPS-stimulated IL-18 expression was detected by RT-PCR. A representative case is illustrated in Figure 3. IL-18 expression was detected in 20 out of 30 nonstimulated normal PBMC samples and all (30/30) of the nonstimulated SLE-PBMC samples. Interestingly, PHA or PWM exhibited inhibitory effects on the expression of IL-18 in both normal PBMC and SLE-PBMC. In patients with SLE, both PWM and PHA exerted significant inhibitory effects on IL-18 expression as compared to the RPMI control ($p = 0.0246$ and $p = 0.0903$, respectively, Mann-Whitney U test; Figure 4). The inhibition of SLE-PBMC was more pronounced by PWM than by PHA. RT-PCR failed to reveal consistent evidence of its expression in normal and SLE-PMN incubated with or with-

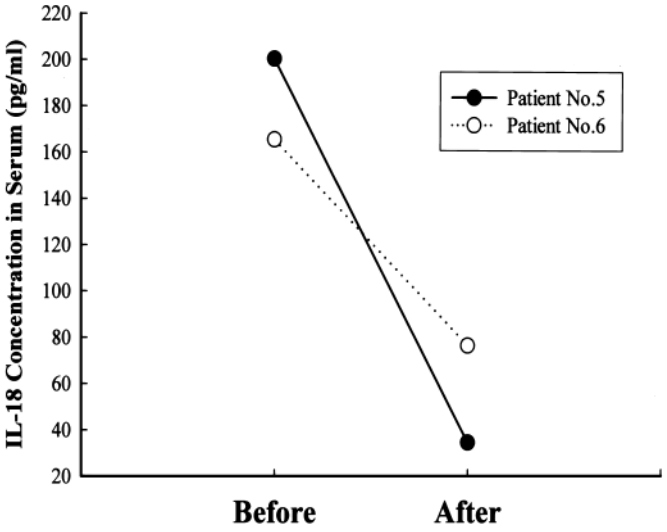

Figure 1. Decrease of IL-18 in serum (by ELISA) in 2 SLE patients receiving large infusions of methylprednisolone (1 g/day for 3 days).

out LPS (data not shown). Unlike PHA or PWM, in many instances, LPS was unable to inhibit the IL-18 expression in SLE-PBMC (Figure 3).

DISCUSSION

IL-18, a member of the IL-1 family, is produced primarily by antigen-presenting cells including macrophage/dendritic cells, Kupffer cells, keratinocytes, and articular chondrocytes[1,9]. It is an important component in innate immunity that facilitates helper T cell activity by skewing the Th1/Th2 ratio in the direction of Th1 in the presence of IL-12 and interferon-γ[10,11]. In this investigation, we have demonstrated that both serum concentration of IL-18 and its expression in PBMC are markedly increased in patients with active SLE, and this increase is correlated to the high activity of lupus disease. This is paradoxical, because IL-18 is an essential stimulator of NK cells, an essential component in the body's defense against microbial infections, especially the intracellular bacterial infections[26]. Shibatomi, *et al* have reported that in autoimmune diseases including SLE, the IL-18 activity is high but NK cell activity is low[15]. The suppressed NK cell activity results from so-called activation-induced cell death exerted by excessive expression and excretion of IL-18. It is conceivable that patients with active SLE are prone to intracellular infections such as *Salmonella enteritides*[27,28] even before the patients receive immunosuppressive therapies. This high activity of Th1 cytokine stimulation in a Th2 cytokine-skewed lupus disease may reflect a compensatory reaction to high Th2 activity rather than a result of overactivity of monocyte/macrophage lineages in active SLE due to increased intracellular infection. Our findings may provide a possible explanation for this important immune defect in patients with SLE. However, the exact correlation of IL-18 to the NK cell activity requires further investigation.

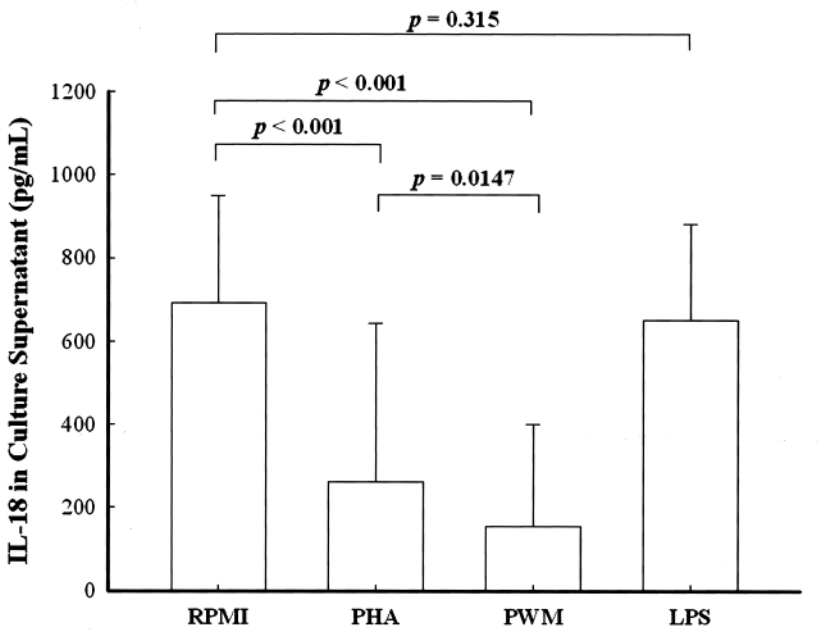

Figure 2. Production of IL-18 by PBMC from patients with SLE, stimulated with phytohemagglutinin (PHA), pokeweed mitogen (PWM), or lipopolysaccharide (LPS), measured by ELISA. Average IL-18 concentrations in culture supernatants were 660.09 ± 290.91 pg/ml in RPMI-1640, 370.39 ± 273.84 pg/ml on PHA stimulation, 219.07 ± 156.00 pg/ml on PWM stimulation, and 581.60 ± 300.48 pg/ml on LPS stimulation. Compared to the RPMI-1640 group, the production of IL-18 from SLE-PBMC was significantly suppressed by PHA ($p < 0.001$) or PWM ($p < 0.001$) (paired t tests).

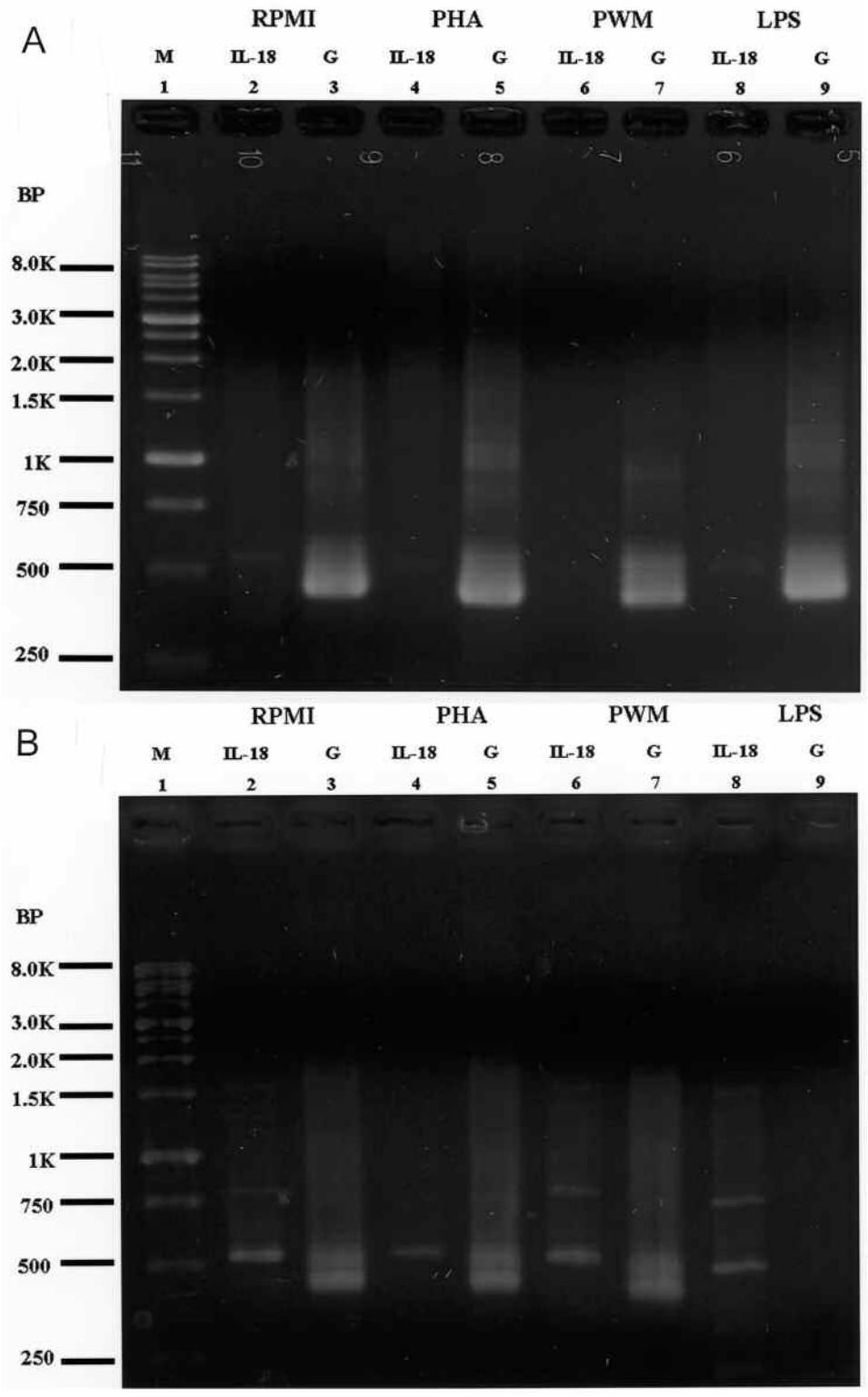

Figure 3. A representative example of RT-PCR to detect expression of IL-18 mRNA in PBMC before and after mitogen or LPS stimulation. Panel A: control; Lane 1: calibration marker (M); Lanes 2 and 3: medium control; Lanes 4 and 5: PHA stimulation; Lanes 6 and 7: PWM stimulation; Lanes 8 and 9: LPS stimulation. Lanes 3, 5, 7, 9 were internal positive GAPDH controls (G). Panel B: SLE patient actively infected with *Staph. aureus*. Lane 1: calibration marker (M); Lanes 2 and 3: medium control; Lanes 4 and 5: PHA stimulation; Lanes 6 and 7: PWM stimulation; Lanes 8 and 9: LPS stimulation. Lanes 3, 5, 7, 9 were internal positive GAPDH controls (G). IL-18 expression was seen in control as well as SLE-PBMC with or without stimulation. PHA and PWM had an inhibitory effect on IL-18 expression in normal PBMC; this inhibitory effect was not clearly seen in SLE-PBMC in this particular example. LPS failed to inhibit IL-18 expression in SLE-PBMC and in normal PBMC.

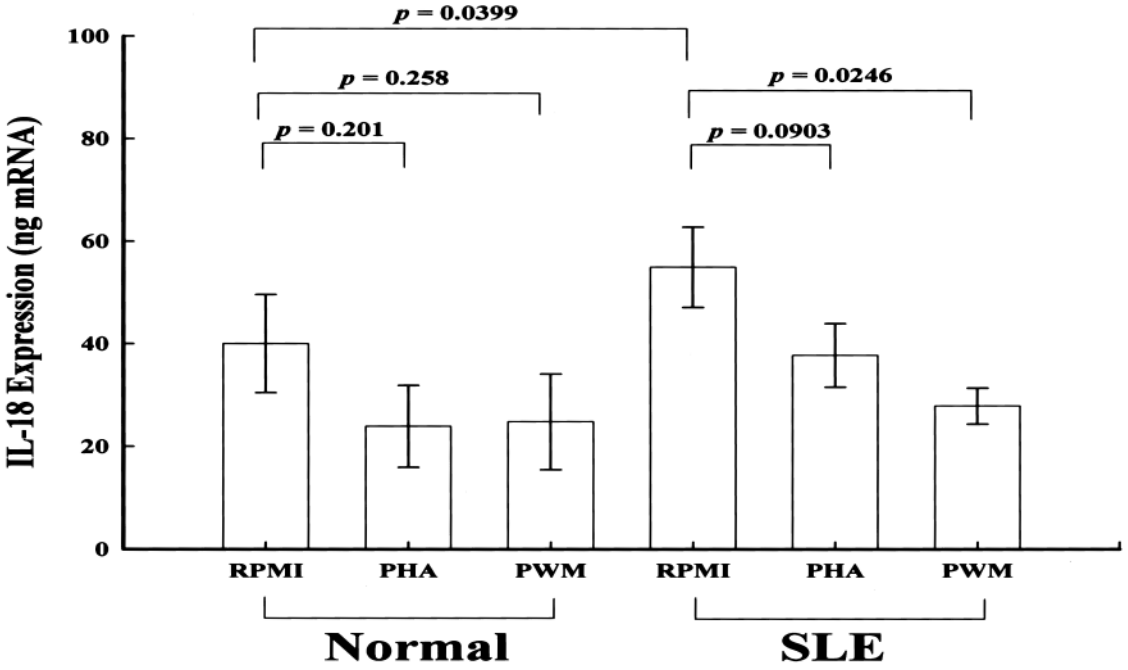

Figure 4. IL-18 transcripts in cultured cells before and after mitogen stimulation (PHA or PWM) in controls and patients with active SLE; semiquantitative measurement. The average equivalent amounts of mRNA were 40.02 ± 9.59 ng (0~176.2) in normal PBMC-RPMI, 23.91 ± 7.95 ng (0~156.2) in normal PBMC-PHA, 24.80 ± 9.29 ng (0~202.4) in normal PBMC-PWM, 54.94 ± 7.81 ng (9.1~177.7) in SLE-PBMC-RPMI, 43.19 ± 6.39 ng (5.9~181.9) in SLE-PBMC-PHA, and 27.85 ± 3.51 ng (1.1~92.2) in SLE-PBMC-PWM. The comparison was made between 2 different stimulation conditions; p values by non-parametric Mann-Whitney U test.

IL-18 can act on the T cells and stimulate Th1 cytokine production in the presence of IL-12[2-5,29,30]. Accordingly, IL-18 is thought to be closely implicated in the pathogenesis of various autoimmune diseases characterized by Th1 hyperactivity. These include rheumatoid arthritis, SLE, adult onset Still's disease, and biliary cirrhosis[6,7,9,11-13]. Our investigation also showed similar results. In 2 patients (Patients 5 and 6), decrease of serum IL-18 paralleled the administration of high-dose methylprednisolone, similarly to a report by Wozniacka, *et al*, whose patients showed lowering of IL-18 with chloroquine treatment[31]. Our results were similar to those showing positive correlation of IL-18 levels with activity of lupus nephritis both in human subjects and in animals[32-35]. Indeed, our findings indicated that this increased activity of IL-18 might be more universal in lupus disease, and not limited to specific organs such as the kidney. It is possible that IL-18 is also implicated in the pathogenesis of other manifestations of SLE (including cytopenia, neuropsychiatric diseases, etc.) in addition to nephritis.

Another interesting point from this investigation is that IL-18 expression seemed markedly suppressed by pokeweed mitogen (PWM), a T-dependent B cell activator. This suppressive effect seemed exaggerated in patients with active SLE (Figure 2 and 4). It is conceivable that active lupus disease is characterized by hyperactivity of B cells with unrestricted production of a variety of autoantibodies. This may also contribute to the downregulation of Thl activity in the late stage of the lupus disease, in addition to the activation-induced NK cell death described by Shibatomi, *et al*[15].

We previously reported that patients with active SLE showed a paradoxical suppression of IL-12 production by PMN in the presence of IFN-γ[17]. The exact biological basis for this phenomenon has not been elucidated. In the present experiments, we showed that the exaggerated IL-18 production in SLE-PBMC, but not PMN, could not be hampered by the bacterial endotoxin LPS. It is possible that with the invasion of bacteria this absence of IL-18 inhibition, associated with the paradoxical IL-12 downregulation in SLE-PMN, may render the phagocytes including PMN or NK cells prone to activation-induced cell death, although normally IL-18, with the help of IL-12, is a potent killer cell activator[36,37]. It is hypothesized that with high IFN-γ production by T cells during bacterial infection the PMN fail to produce IL-12, and NK cells undergo activation-induced cell death or apoptosis. This may ultimately lead to the overwhelming opportunistic infections frequently observed in the late stage of active SLE.

In conclusion, the expression and production of IL-18 in patients with active SLE is high and is correlated with disease activity. IL-18 expression and production may be enhanced in the initial stage of the disease and be suppressed after prolonged hyperactivity of the B cells. The

exact correlation of NK cell activity with the production of IL-18 in lupus disease requires further investigation.

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