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Expression of interleukin-12 and its signaling molecules in peripheral blood mononuclear cells in systemic lupus erythematosus patients

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Objective To determine the in vitro expression of interleukin-12 (IL-12) and its effect on signal transducers and activators of transcription (STAT) signaling molecules in peripheral blood mononuclear cells (PBMCs) in patients with systemic lupus erythematosus (SLE).

Methods Peripheral blood mononuclear cells in 39 patients with definite systemic lupus erythematosus and 11 healthy volunteers were collected. Expression of IL-12 P40mRNA in PBMCs was determined with reverse transcription-polymerase chain reaction (RT-PCR). Quantity of IL-12 protein supernatant was measured by enzyme-linked immunosorbent assay (ELISA). The levels of phosphorylated STAT3 and STAT4 signaling molecules in PBMCs were detected by immunoblot.

Results Levels of IL-12 protein and mRNA expression in patients with active or inactive SLE were significantly higher than those in controls. Phytohemagglutinin (PHA) may promote the expression of IL-12. IL-12 alone induced the phosphorylation of STAT3 and STAT4 in PBMCs from patients with SLE，especially in active SLE. However it had no obvious effect on normal PBMCs. Phosphorylated STAT3 and STAT4 might be observed in normal PBMCs treated with IL-12 plus PHA.

Conclusion IL-12 is produced aberrantly in patients with SLE. IL-12 might exert its biological role in SLE via the aberrantly phosphorylated STAT3 and STAT4 signaling molecules.

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Systemic lupus erythematosus (SLE) is an autoimmune disorder which is characterized by the impaired immunoregulation reflected in abnormal humoral and cellular immune responses during the active state. Constitutive abnormalities in T and B cells play an important role in this disease. Both biochemical and functional abnormalities affecting cytokine production，antigen/mitogen driven proliferation and signal transduction pathways in lupus T and B cells have been reported. Abnormalities in T and B lymphocytes and excessive autoantibody production are the features of this disease.[1-3] Recent studies showed that a large quantity of interleukin-12 (IL-12) could be generated in vitro by T and B lymphocytes[4] and monocytes following stimulation with phytohemagglutinin (PHA) or lipopolysaccharide (LPS)，which mediate the regulation of cell proliferation and differentiation and autoantibody production by feedback.[5] IL-12 can induce phosphorylation of signal transducers and activators of transcription (STAT)，STAT3 and STAT4，signaling molecules in T cells pretreated with IL-2 or PHA. However，it is still not clear whether IL-12 is aberrantly produced in SLE and how it exhibits its biological role through signaling pathways.

In this study，we present an evidence that the expression of IL-12 is aberrant in SLE and IL-12 alone induces the phosphorylation of STAT3 and STAT4 in peripheral blood mononuclear cells (PBMCs) in SLE，but has no effect on normal PBMCs.

METHODS

Patients

Thirty-nine patients who fulfilled at least 4 criteria for SLE

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established in 1982 by the American College of Rehumatology were studied. Of them, 37 were women and 2 were men. At the time of the study, the mean age was 30 ± 7 years (range 18 – 46 years) and mean disease duration from first symptoms was 6 ± 4 years. Activity of SLE was estimated using the SLE Disease Activity Index (SLEDAI).[6] According to the active index, patients were divided into two groups. Twenty-one patients were in the active SLE group, with a mean SLEDAI score of 15 ± 6 (range 9 – 20). Eighteen patients were in the inactive SLE group, with a mean SLEDAI score of 2 ± 7 (range 3 – 8). The control group included 11 healthy volunteers (10 women and 1 man) who had a mean age of 30 ± 4 years (range 25 – 33 years). None of them were receiving immunosuppressive drugs or blood products within three weeks before taking blood samples.

Materials

All reagents were purchased from Sigma Company, if not otherwise cited. Polyclonal rabbit antibodies against STAT3, STAT4 (C-20 and L-18) and Py-20 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ECL kits were from Amersham (Arlington Heights, IL). The total RNA isolation kit and reverse transcription-polymerase chain reaction (RT-PCR) kit were purchased from Boehringer Mannheim (GE, Germany). Ficoll-Hypaque was from Pharmacia (Pharmacia, Uppsala, Sweden). PHA and rIL-12 were provided by Pepro Tech (Pepro Tech, England).

Cell culture

Ten milliliters of peripheral blood were diluted with 10 ml of phosphate buffered saline (PBS), layered carefully over 7.5 ml of Ficoll-hypaque and centrifuged at 400 g for 30 minutes at room temperature. 250 g hypaque was kept for 10 minutes to remove other types of cells, and so PBMCs was got. PBMCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and further incubated for the indicated times with PHA (2 mg/ml) or rIL-12 (10 ng/ml). PBMCs were incubated at 37℃ in 5% CO_2 in a humidified incubator. 2×10^6 cells were incubated for 48 hours to harvest supernatants. All experiments were repeated at least twice, using cells from different donors.

RT-PCR analysis

Total RNA preparation cells were washed twice with 10 ml PBS and suspended in 1 ml of denaturing solution for RNA preparation using the total RNA isolation kit. RNA was quantified by absorbance at 260 nm and purity was checked by 260/280 nm absorbance and by agrose-formaldehyde gel electrophoresis after staining with ethidium bromide. Expression of IL-12 mRNA was detected by RT-PCR using primers. Primers were synthesized according to the cDNA sequence,[7] which generated a 272 bp fragment: sense, CCA-CAT-TCC-TAC-TTC-TC; antisense, GTC-TAT-TCC-GTT-GTG-TC. β-actin primers were used to perform RT-PCR, which generated a 587 bp fragment: sense, CAA-AGG-CCA-ACC-GCC-GGG-AGA-AAG-TGA; antisense, AGG-GTA-CAT-GGT-GGT-GCC-GCC-AGA-C.

The cDNA were made by using an RT-PCR kit. Synthesis was carried out in a 50 ml reaction volume containing 4 μg of total RNA. Amplification was performed in a DNA thermal cycler (Perkin Elmer, Germany) as follows: initial denaturation at 94℃ for three minutes, 35 cycles of amplification (94℃ for 30 seconds, 54℃ for 45 seconds, and 68℃ for 60 seconds), and final extention at 68℃ for 10 minutes. These fragments were readily distinguishable by ethidium bromide staining after electrophoretic migration in 1.5% agarose gels. Area percentages of IL-12 mRNA/β-actin mRNA were analysed by a computer picture analysis system (UVP GDS 7600, Germany).

Quantitative measurement of IL-12

In order to determine IL-12 in the supernatant of PBMCs activated in vitro with PHA, a commercial enzyme-linked immunosorbent assay (ELISA) kit was used (Pepro Tech, England) according to the manufacturer's instructions.

Immunoblot analysis

Cells were pretreated with protein kinase inhibitors for 0.5 hours. Cells were further cultured for 1 hour in the presence or absence of inhibitors. 5×10^6 cells were then washed with phosphate buffered saline three times and incubated in radioimmune precipitation assay (RIPA) lysis buffer [0.5% Non nidet P-40, 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L ethylene glycol tetraacetate (EGTA), 1 mmol/L phenylmethlsulfonyl fluoride, 100 mmol/L leupeptin, 100 mmol/L pepstain A][8,9] for 10 minutes at 4℃. The cell lysate was centrifuged at 1300 g for 10 minutes and the supernatant was used as cytoplasmic extract. Protein concentration was determined using the Bradfold assay kit from Bio-Rad.

For immunoprecipitation, 20 μg extract was transfered to a microcentrifuge tube, and 5 μl monoclonal antibody (anti-STAT3, anti-STAT4, Santa Cruz Biotecnology, Santa Cruz, CA, USA) was added, incubated for 1 hour at 4℃. Then 20 μl protein A-Agrose was added and incubated for 10 hours. Immunoprecipitate was collected by centrifugation at 2500 r/min for 5 minutes at 4℃. Samples were then boiled for 3 minutes, and the cell lysate was separated by electrophoresis through 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The blot was probed with the secondary

antibody (anti-STAT3, anti-STAT4, Santa Cruz Biotecnology, Santa Cruz, CA, USA) for 1 hour at room temperature. Chemiluminesence detection (ECL, Amersham) was carried out according to the manufacturer's specifications using peroxidase conjugated anti-rabbit immunoglobin (1∶1000, Santa Cruz Biotecnology, Santa Cruz, CA, USA). After the nitrocelluose membranes were stripped, phosphorylation of STAT3 and STAT4 was determined using the blot probed with py-20 antibody.

RESULTS

Increased IL-12 mRNA levels in PBMCs from patients with SLE

PBMCs from control and SLE groups were cultured with PHA or PHA plus IL-12. Representative results of RT-PCR for IL-12 P40 are shown in Fig. 1. Following stimulation of PBMCs with PHA, IL-12 P40 mRNA expression was higher in both active and inactive SLE PBMCs than in controls ($P < 0.05$). IL-12 P40 mRNA expression in PBMCs treated with IL-12 plus PHA was significantly higher in both active and inactive SLE than in controls ($P < 0.01$). IL-12 P40 mRNA expression was greater in active SLE than in inactive SLE ($P < 0.01$, Fig. 2).

Increased IL-12 protein levels in PBMCs from SLE patients

Levels of IL-12 measured by ELISA were much higher in supernatants from PBMCs of active or inactive SLE than in those of controls, no matter whether they were stimulated with PHA or not (Table, $P < 0.01$).

Table. Concentration of IL-12 in supernatants of cultured PBMCs

Group	Number	Control (pg/ml)	PHA (pg/ml)
Normal control	11	29.8 ± 9.1	34.2 ± 11.2
Active SLE	21	174.2 ± 12.3*	209.6 ± 23.7*
Inactive SLE	18	67.4 ± 10.7*	82.8 ± 21.3*

Elispot assays for the detection of IL-12-secreting cells were performed in PBMCs cultured with or without PHA, as described above. Values are expressed as $\bar{x} \pm s$. * $P < 0.01$, versus normal control.

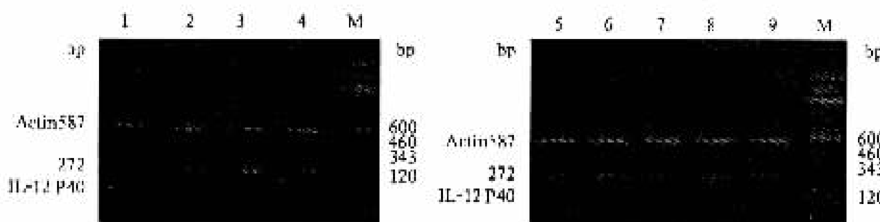

Fig. 1. RT-PCR analysis of IL-12 expression in normal and SLE (active or inactive) PBMCs. Total RNA was isolated from PBMCs, and RT-PCR for IL-12 and β-actin was performed. PBMCs from the normal control (Lanes 1 – 3), the inactive SLE (Lanes 4 – 6), or the active SLE (Lanes 7 – 9) were stimulated with nothing, PHA (2 mg/ml) and PHA (2 mg/ml) plus IL-12 (10 ng/ml), respectively. We present the ethidium bromide staining of RT-PCR products analyzed on a 1.5% agarose gel. Amplification of the β-actin gene was used as an internal control. M: marker.

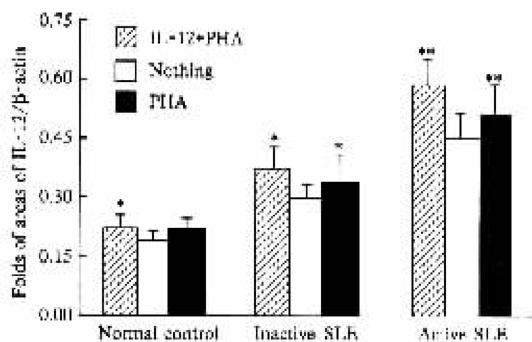

Fig. 2. Comparison of expression of IL-12 P40 mRNA among these groups. PBMCs from SLE patients and from control were cultured with nothing, PHA (2 mg/ml), or IL-12 (10 ng/ml) plus PHA (2 mg/ml). Results are expressed as $\bar{x} \pm s$. Eleven healthy volunteers and 39 SLE patients were tested. * $P < 0.05$, ** $P < 0.01$ versus control (Man-Whitney U-test).

IL-12 phosphorylated STAT3 and STAT4

IL-12 clearly activated STAT3 and STAT4, but it induced only weak phosphorylation of STAT3 in active SLE. It did not induce significant phosphorylation of STAT3 in normal PBMCs, whereas PHA induced weak tyrosine phosphorylation of STAT3 and STAT4. IL-12 induced tyrosine phosphorylation of STAT3 and STAT4 in active SLE and the level of phosphorylation was much stronger than that produced by IL-12 in PBMCs from inactive SLE, but weaker than that produced by IL-12 plus PHA-treated PBMCs in active SLE. Stripping and reprobing membranes were sequentially carried out with antibodies to STAT3 and STAT4, which were used as internal controls, so the experiment was ensured to be done at the same conditions.

DISCUSSION

IL-12 is recently identified as inflammatory cytokine produced mainly by T and B lymphocytes and monocytes. It promotes the production of IFN-γ by T lymphocytes and is necessary for differentiation of Th naive cells to Th1 cells.[10]

Th1 type immune response modulates the production of IgG2a, IgG3 anti-dsDNA and anti-Sm autoantibodies.[11] IL-12 is a P70 heterodimeric cytokine formed by P35 and P40 subunits. P35 is produced by many cell types, whereas P40 is expressed specifically by IL-12 producing cells.[12] Similar results were obtained in our previous experiments. The determination of P40 subunits may reflect the expression level of IL-12.

IL-12 can induce the initiation of autoimmune disease and evidence shows that IL-12 can accelerate the development of SLE.[12-14] In our study, IL-12 in SLE patients was higher than in controls. Moreover, the level of IL-12 in active SLE was much higher than in inactive SLE, which suggests that PBMCs are aberrant in SLE patients, for they secret large amounts of IL-12. On the other hand, PHA significantly promoted PBMCs to produce IL-12 in SLE, whereas it had no obvious effect on PBMCs in controls. This shows that PBMCs are activated in SLE and have a sensitive response to PHA or inflammatory stimulations.

Our experiments showed that IL-12 mRNA expression in SLE patients was higher than that in controls, possibly resulting from an activated IL-12 gene in SLE. It also suggests that PBMCs are activated in SLE, and they promote IL-12 gene expression. These results are consistent with the fact that IL-12 mRNA expression and protein expression are greater in SLE. IL-12 mRNA expression was greater after stimulation with PHA or IL-12 plus PHA, suggesting that there was a direct link between aberrant activation of PBMCs and IL-12 production. Although PBMCs were normal in the controls, yet they may be activated after pretreatment with PHA. IL-12 exhibited its biological role via the PHA activated PBMCs in the controls. IL-12 alone had minor effect on normals, differing from IL-2, IFN-γ and other cytokines that have direct effects on PBMCs. Whether there were special signaling pathways in IL-12 signal transduction in PBMCs, especially in SLE patients, may be related to STAT3 and STAT4 signaling molecules in SLE PBMCs.

Signaling by many cytokines involves phosphorylation of proteins known as STAT, forming dimeric complexes and bind specific DNA sequences.[15-17] Most known STATs bind IFN-γ-activated (GAS) motifs containing the consensus sequences: TTNCNNNAA.[18]

IL-12 and PHA also activate both distinct and overlapping components of the JAK/STAT signaling pathway in activated T cells.[19-21] The importance of JAK /STAT signaling in mediating the functional effects of IL-12 has been demonstrated by the phenotype of STAT4 knockout mice.[22] Recently, IL-12 has been found to induce tyrosine phosphorylation of STAT3 and STAT4 in Th1 cells. In mice lacking STAT4, all the major functional effects of IL-12 on T and NK cells are markedly inhibited.[23] Cytokines can also induce the serine phosphorylation of STATs. Tyrosine phosphorylation of STATs is required for dimerization, nuclear translocation and DNA binding and is therefore necessary to induce STAT-responsive gene transcription.

IL-12 induces strong tyrosine phosphorylation of STAT4 and variable weak phosphorylation of STAT3 in active SLE. Differential phosphorylation and consequent different activation of both separate and overlapping STAT proteins by PHA and IL-12 may provide a molecular basis for the similarities and differences in the activation of these stimulators for PBMCs in SLE.

This study indicates that PBMCs in SLE patients are aberrantly activated, and IL-12 mRNA expression and protein production are enhanced in PBMCs, which promote PBMC proliferation and differentiation via STAT3 and STAT4 signaling pathways.[24] The stimulation of PBMC by IL-12 may result in secretion of autoantibodies. These observed IL-12 induced events could be involved in the pathogenesis of SLE and provide new clues for understanding the molecular bases of autoimmunity.

REFERENCES

1. Klinman DM, Steinberg AD. Inquiry into murine and human lupus. Immunol Rev 1995;144:157-193.
2. Foster MH, Cizman B. Nephritogenic autoantibodies in systemic lupus erythematosus: immunochemical properties, mechanisms of immune deposition, and genetic origins. Lab Invest 1993;69:494-507.
3. Jongstra-Bilen J, Vukusic B, Boras K, et al. Resting B cells from autoimmune lupus-prone New Zealand Black and (New Zealand Black x New Zealand White) F1 mice are hyper-responsive to T cell-derived stimuli. J Immunol 1997;159:5810-5820.
4. Wolf SF, Sieburth D, Sypek J. Interleukin 12: a key modulator of immune function. Stem Cells 1994;12:154-168.
5. Nakajima A, Hirose S, Yagita H, et al. Roles of IL-4 and IL-12 in the development of lupus in NZB/W F1 mice. J Immunol 1997;158:1466-1472.
6. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-1277.
7. Mattei S, Colombo MP, Melani C, et al. Expression of cytokine/growth factors and their receptors in human melanoma and melanocytes. Int J Cancer 1994;56:853-857.
8. Yamamoto K, Miura O, Hirosawa S, et al. Binding sequence of STAT4: STAT4 complex recognizes the IFN-gamma activation site (GAS)-like sequence (T/A) TTCC (C/G) GGAA (T/A). Biochem Biophys Res Commun 1997;233:126-132.
9. Yu CR, Lin JX, Fink DW, et al. Differential utilization of Janus kinase-signal transducer activator of transcription signaling pathways in the stimulation of human natural killer cells by IL-2

and IFN-alpha. J Immunol 1996；157：126-137.
10. Suzuki Y，Koyanagi Y，Tanaka Y，et al. Determinant in human immunodeficiency virus type 1 for efficient replication under cytokine-induced CD4+ T-helper 1（Th1）- and Th2-type conditions. J Virol 1999；73：316-324.
11. Takahashi S，Fossati L，Iwamoto M，et al. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. J Clin Invest 1996；97：1597-1604.
12. Kubin M，Chow JM，Trinchieri G. Differential regulation of interleukin-12（IL-12），tumor necrosis factor alpha，and IL-1 beta production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood 1994；83：1847-1855.
13. Liblau RS，Singer SM，McDevitt HO. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 1995；16：34-38.
14. Blotta MH，DeKruyff RH，Umetsu DT. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. J Immunol 1997；158：5589-5595.
15. Shuai K，Horvath CM，Huang LH，et al. Interferon activation of the transcription factor STAT91 involves dimerization through SH2-phosphotyrosyl peptide interactions. Cell 1994；76：821-828.
16. Xu X，Sun YL，Hoey T. Cooperative DNA binding and sequence-selective recognition conferredby the STAT amino-terminaldomain. Science 1996；273：794-797.
17. Vinkemeiier U，Cohenn SL，Moarefi I，et al. DNA binding of in vitro activated STAT1 alpha，STAT1 belta and truncated STAT1：interaction between NH2-terminal domains stabilize binding of two dimers totandem DNA sites. EMBO J 1996；15：2425-2433.
18. Tessitore A，Pastore L，Rispoli A，et al. Two gamma-interferon-activation sites（GAS）on the promoter of the human intercellular adhesion molecule（ICAM-1）gene are required for induction of transcription by IFN-gamma. Eur J Biochem 1998；258：968-975.
19. Bright JJ，Du C，Sriram S. Tyrphostin B42 inhibits IL-12-induced tyrosine phosphorylation and activation of Janus kinase-2 and prevents experimental allergic encephalomyelitis. J Immunol 1999；162：6255-6262.
20. Kilic M，Flossmann E，Flossmann O，et al. Jeg-3 human horiocarcinoma-induced immunosuppression：downregulation of interleukin-2，interleukin-2 receptor alpha-chain，and its Jak/Stat signaling pathway. Am J Reprod Immunol 1999；41：61-69.
21. Klein JL，Fickenscher H，Holliday JE，et al. Herpesvirus saimiri immortalized gamma delta T cell line activated by IL-12. J Immunol 1996；156：2754-2760.
22. Wild J，Grusby MJ，Schirmbeck R，et al. Priming MHC-I-restricted cytotoxic T lymphocyte responses to exogenous hepatitis B surface antigen is CD4+ T cell dependent. J Immunol 1999；163：1880-1887.
23. Kaplan MH，Sun YL，Hoey T，et al. Impaired IL-12 responses and enhanced development of Th2 cells in STAT4-deficient mice. Nature 1996；382：174-177.
24. Jelinek DF，Braaten JK. Role of IL-12 in human B lymphocyte proliferation and differentiation. J Immunol 1995；154：1606-1613.

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