行政院國家科學委員會專題研究計畫　成果報告

IL-12家族細胞激素在CD4+T細胞中的JAK-STAT訊號傳遞及其引發基因表現之研究

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-040-067-
執行期間：93年08月01日至94年07月31日
執行單位：中山醫學大學醫學影像技術學系

計畫主持人：李尚熾

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94年10月31日

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計畫中文摘要

IL-12 是調控Th1 發展與細胞性免疫的關鍵細胞激素，它是由p40 與p35 次單元組成的異二聚體。在CD4$^+$ T細胞中，IL-12 透過JAK-STAT訊號傳遞途徑使STAT4 酪氨酸磷酸化成為活化的轉錄因子而引發相關基因表現，如γ干擾素基因，使細胞分化為Th1 活化毒殺性T細胞及巨噬細胞。正因為IL-12 在免疫調節上的重要性，許多研究指出IL-12 可以有效治療病毒感染、類風濕性關節炎等某些自體免疫疾病、甚至是癌症。最近，透過基因序列資料之比對，科學家們找出了和IL-12p35 結構相類似的兩個蛋白質p19 和p28。p19 可以和IL-12p40 結合而形成IL-23，而p28 則和EBI3 結合形成IL-27。IL-27、IL-12 與IL-23 因為結構上的相似性而被視為同一家族的細胞激素，它們的功能也被證實皆可調控Th1 發展，但是和IL-12 比較起來，IL-23 只作用在記憶細胞，而IL-27 主要作用於Th1 分化的初始階段，因此這三個細胞激素似乎對Th1 的發展有階段性的貢獻。在治療應用上，而我們去年也報告指出IL-23 和IL-12 一樣也同樣具有抗癌與抗轉移的功效，並可刺激小鼠對癌細胞產生免疫記憶。然而到目前為止，我們並不清楚IL-23 或是IL-27 在CD4$^+$ T細胞中的訊號傳遞途徑，及它們調控Th1 分化的分子機制。本計畫以短干擾RNA(siRNA)抑制CD4$^+$/CD45RB^{low} T細胞中STAT4 的表現，發現會因此阻斷細胞對IL-23 之反應，顯示STAT4 對此細胞激素訊號傳遞過程中的必要性。

關鍵字：細胞激素，訊號傳遞

計畫英文摘要

Interleukin-12 (IL-12), which is composed of a p35 and a p40 subunit, is a proinflammatory natural-kill cell-stimulating, Th1-inducing and Th1-maintaining cytokine, which promote cell-mediated immunity. These biological functions make IL-12 a potent therapeutic agent for virus infection, malignant diseases, and autoimmune diseases. The JAK-STAT pathway has been demonstrated to be activated by IL-12 and play an important role in IL-12 signaling. IL-12-dependent synthesis of IFN-γ induction, T cell proliferation, and natural killer activation requires the activity of the transcription factor STAT4. Recently, it is reported that p40 can be covalently linked to a p35-related protein p19. This heterodimer is known as IL-23 and has activities on memory T cells. IL-27 is a heterodimer composed of the p40-related protein EBI3 (Epstein–Barr virus-induced gene 3) and the p35-related protein p28. IL-27 is involved in early Th1 initiation.

However, the molecular mechanism underlay the signal transduction of IL-23 or IL-27 remains unclear. It will be important to determine how IL-23/IL-27 integrates into the IL-12 family with regard to its function in Th1 development. In this study, the role of STAT4 in IL-23 signal transduction had been examined.

Keywords: IL-23, STAT4, signal transduction

報告內容

The regulation of the differentiation of naïve T helper (Th) 0 cells into either Th1 or Th2 cells is a critical process when Th0 cells encounter antigen. Understanding the underlying molecular mechanism of Th1/Th2 differentiation may provide a conceptual framework in developing immune modulation therapies against autoimmune diseases, allograft rejection, allergic diseases, and malignancies. The dominant factors that control the differentiation program are cytokines. Th0 cells activated in the presence of interleukin-4 (IL-4) differentiate into Th2 cells and promote humoral (antibody-mediated) immunity. In contrast, IL-12 promotes IFN-γ production and the differentiation of Th0 cells into Th1 cells, resulting in enhanced cellular immunity (*1*).

IL-12 is a heterodimeric molecule composed of a 35-kDa α subunit (p35) and a 40-kDa β subunit (p40) linked by a disulphide bridge to form the bioactive 70kDa cytokine (*2, 3*). The biological activities of IL-12 are mediated by a high-affinity IL-12 receptor, consisting of a β1 chain (IL-12Rβ1) and a β2 chain (IL-12Rβ2) (*4*). Upon binding of IL-12, both chains of the IL-12 receptor heterodimerize, and activate the associated Janus kinases (JAKs), TYK2 and JAK2 (*5*). TYK2 interacts with the IL-12Rβ1 and JAK2 interacts with the IL-12Rβ2 (*6*). Furthermore, JAKs phosphorylate the IL-12 receptor on tyrosines located in the intracellular domain. The phosphorylated regions are binding sites for transcription factors termed Signal Transducers and Activators of Transcription (STAT). These phosphorylated receptors recruit STATs to the complex (*5, 7, 8*), which, after phosphorylation and dimerization, are transported to the nucleus, where they regulate transcription of a number of genes. IL-12 binding to its receptor results in activation of three members of the STAT family, that is, STAT1, STAT3, and STAT4 (*7-11*). The specific cellular effects of IL-12 including the induction of IFN-γ and the differentiation of Th1 cells are due mainly to activation of STAT4, as indicated by the fact that deletion of the STAT4 gene in knockout mice results in defective responses specific to IL-12 (*12, 13*).

The recently discovered cytokine IL-23 consists of a heterodimer of the IL-12 p40 subunit and a novel 19-kDa protein, termed p19 (*14*). Structurally, p19 is distantly related to IL-6, G-CSF, and the IL-12 p35 subunit (*14*). Although p19 is expressed in various tissues and cell types, per se it lacks biological activity (*14*) and only becomes biologically active when complexed with p40, which is normally secreted by activated macrophages and dendritic cells. Mice that are transgenic for IL-23p19 have a marked phenotype of premature death and multi-organ inflammation characterized by lymphocyte and macrophage infiltration (*15*). Adoptive

transfer experiments of transgenic bone marrow cells into normal host transfer the disease phenotype, demonstrating that this inflammatory phenotype results from the overexpression of IL-23 in hematopoietic cells (*15*). These results indicate that IL-23 shares the pro-inflammatory biological properties of its homologous cytokines IL-12 (*15*). One of the shared actions of IL-12 and IL-23 is their proliferative effect on T cells (*14*). However, clear differences exist in the T cell subsets on which these cytokines act. IL-23 preferentially induces proliferation of, and IFN-γ production by, memory T cells as opposed to naïve T cells, whereas the effects of IL-12 are mainly restricted to naïve T cells (*14*). The differential response of naïve and memory T cells to IL-23 correlates well with the preferential expression of the specific IL-23 receptor on memory T cells (*16*).

IL-23 binds to a receptor that is formed by IL-12Rβ1 and a novel IL-23R subunit (*16*). The similarity between IL-23R and IL-12Rβ2 is further highlighted by shared use the same JAK–STAT signaling molecules (JAK2, TYK2, STAT1, STAT3, STAT4 and STAT5) (*16, 17*). The intracellular signaling pathways of IL-12 and IL-23 are very similar, but they differ in the extent to which they result in the activation of STAT4 (*17, 18*). IL-23 induces much less phosphorylation of STAT4 than does IL-12, and the majority of the activated STAT4 heterodimerize with STAT3. This contrasts signal transduction following IL-12 stimulation, which is characterized by a predominance of STAT4 homodimers, perhaps indicating that IL-12 and IL-23 may act on different target genes (*16*). However, the significance of these differences in STAT4 interactions following activation by IL-12 or IL-23 is unknown.

siRNA is a powerful reverse genetic tool which can induce sequence-specific gene silencing through RNA-mediated interference (RNAi) in mammalian cells. RNAi is an evolutionarily conserved mechanism of post-transcriptional gene silencing, triggered by double-stranded RNA that is homologous to the silenced gene (*19, 20*). In RNA silencing, endogenously synthesized or exogenously applied dsRNA is cleaved into 21- to 23-nucleotide fragments by Dicer, a member of the RNase III family. These small RNA fragments, also called siRNAs, associate with a nuclease complex, named RNA-induced silencing complex (RISC) and guide this complex to find and destroy the homologous cellular mRNAs by complementary base pairing (*21-23*). RNAi has been used as a tool to study gene function in several model organism, including plants, *Caenorhabditis elegans*, and *Drosophila*, where large dsRNA efficiently blocks gene expression and is several orders of magnitude more efficient than antisense or ribozyme treatment (*20, 24, 25*). In year 2001, Elabshir and coworkers reported that gene-specific suppression in mammalian cells can be achieved by use of synthetic short (21-nucleotide in length) siRNA (*26, 27*). To date, the application of siRNA has been extended to suppress gene expression of viral oncoproteins, signaling molecules, CD4/CD8 cell surface marker, and cytokine in a wide variety of primary cells including

T cells and dendritic cells (*28-30*).

In this study, we had induced a transient suppression of STAT4 by transfect $CD4^+$/$CD45RB^{low}$ memory T cells with specific anti-STAT4 siRNA. It is found that the STAT4-silenced memory T cells would not response to IL-23. These results demonstrated that STAT4 may play an essential role in IL-23 signal transduction.

Research Design and Methods

Vector construction: cDNA encoding murine p40, p35, p19, EBI3, p28 was obtained by reverse transcription and PCR amplification of RNA derived from the mouse macrophage cell line A3.1A7. To facilitate gene cloning into retroviral vectors, an overlap PCR strategy was used to generate scIL-12, scIL-23, and scIL-27 cDNA in the p40-p35, p40-p19, and EBI3-p28 orientation, respectively (*31*). The final amplified product containing the linker sequence was ligated into pCR-Blunt (Invitrogen, Carlsbad, CA) for sequencing and subsequent cloning. The pBlunt plasmid was digested at the *Xho*I and *Hin*dIII sites to release the DNA fragment, which was then inserted into the pLNCX2 retroviral vector (BD Clontech, Palo Alto, CA) to produce the plasmid pLNCX/scIL-12, pLNCX/scIL-23, pLNCX/scIL-27 (Fig. 1*A*).

Expression of single-chain cytokine proteins: 3T3 cells ($1x10^6$) were transiently transfected with 5 μg of pLNCX/scIL-12, pLNCX/scIL-23, or pLNCX/scIL-27 using lipofectamine 2000. After incubation, the supernatants were harvested and affinity purified by rat anti-mouse IL-12p40 mAb- or rat anti-mouse p28 mAb-coupled sepharose beads (Fig. 1*B*).

Biofunctional assay of single-chain cytokine proteins: For the cytokine functional assay, $CD4^+$ T cells from the spleen and lymphnodes of naive mice were enriched using nylon wool followed by selection with a magnetically labeled anti-CD4 mAb (L3T4; Miltenyi Biotec, Auburn, CA) using the MACS system (Miltenyi Biotec). FACS analysis of the purified $CD4^+$ T cells showed ~95% purity (data not shown). The purified $CD4^+$ T cells ($2x10^4$/well) were then cultured for 5 days in wells coated with anti-CD3 mAb 145.2C11 (CRL-1975; American Type Culture Collection) in medium containing serial dilutions of test samples. To measure IFN-γ release, the cell-free supernatants were prepared and assayed using a sandwich ELISA, with the capture mAb being R4-6A2 (rat IgG1; BD PharMingen) and the detection biotinylated mAb being XMG1.2 (rat IgG1; BD PharMingen). The ELISA was performed according to the manufacturer's instructions and has been described previously (*31*). To measure proliferation, the cells were pulsed for 18 h with [^{3}H]thymidine (1 μCi per well) and the incorporated radioactivity was determined as described previously (*31*).

To investigate the functional activities of the fusion cytokines, the conditioned medium from pLNCX2-, pLNCX/scIL-12-, or pLNCX/scIL-23-transfected 3T3 fibroblasts was tested for its ability to stimulate purified $CD4^+$T cells in the presence of platebound anti-CD3 mAb.

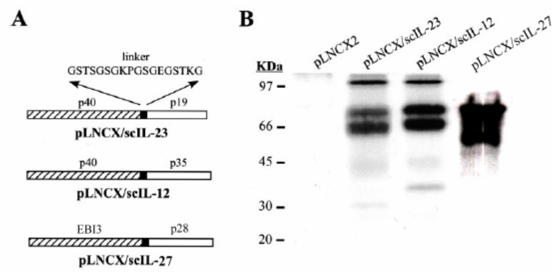

Figure 1. Construction and expression of single chain IL-12, IL-23, IL-27. *A*, Schematic diagram of pLNCX/scIL-12, pLNCX/scIL-23, and pLNCX/scIL-27. The single-chain proteins scIL-23 and scIL-12 were in the p40-p19 and p40-p35 orientation, separated by a flexible 18-aa peptide linker. *B*, Proteins in the supernatant were immunopurified and analyzed on an SDS polyacrylamide gel.

As shown in Fig. 2*A*, the scIL-23-containing supernatant stimulated the proliferation of $CD4^{+}T$ cells in a dose-dependent manner and to the same degree as the scIL-12-containing supernatant. In contrast, the scIL-23-containing supernatant failed to induce IFN-γ production, whereas the scIL-12-containing supernatant strongly stimulated IFN-γ secretion (Fig. 2*B*). The pLNCX2 plasmid failed to stimulate the proliferation of $CD4^{+}T$ cells and IFN-γ secretion.

siRNA design and synthesis: The selection of the coding sequence for siRNA was analyzed by BLAST research to ensure that they did not have significant sequence homology with other genes. The individual motif is 21nt long and corresponds to nucleotides 121–141 of the STAT1 coding region or to nucleotides 136–157 of the STAT4 coding region. siRNA will be commercial synthesized and HPLC purified by MWG (Germany).

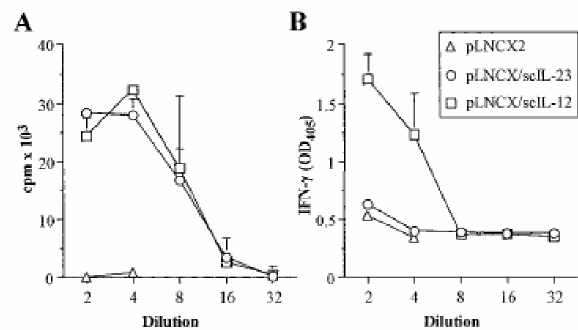

Figure 2. Functional assay of scIL-12 and scIL-23. 3T3 cells were transfected with various proviral plasmids for 48 h, and then the culture supernatants were collected for the functional assay. Purified mouse $CD4^{+}T$ cells were cultured for 5 days with serial dilutions of transfected cell culture supernatants in the presence of plate-bound anti-CD3 mAb, and then cell proliferation was assessed by $[^{3}H]$thymidine incorporation (*A*) and the IFN-γ content was determined by ELISA (*B*). The values are presented as the mean±SD for triplicate wells. The data are representative results of three independent experiments.

For annealing of siRNA, 20 mM complementary single stranded RNAs will be incubated in annealing buffer (20 mM Tris-HCl, pH 7.5; 10 mM MgCl; and 50 mM NaCl) for 1 min at 90℃ followed by 1 h at 37℃. anti-STAT siRNA were transfected into $CD4^{+}T$ cells. The ability of these siRNA to suppress their cognate gene expression and protein production were tested by quantitative PCR and immunoprecipitation analysis, respectively. Anti-luciferase siRNA were included in all experiments to serve as a control. Methods are described below in detail.

Isolation and transfection of $CD4^+T$ Cells: Naïve and memory $CD4^+T$ cells from the lymphnodes of mice were highly enriched by using Mouse Naive T Cell $CD4^+/CD62L^+/CD44^{low}$ Column Kit and Mouse Memory T cell $CD4^+/CD62L^-/CD44^{high}$ Column Kit (R&D), respectively. FACS analysis of the purified $CD4^+$ T cells showed ~92% purity (Fig. 3B). The purified $CD4^+T$ cells ($2x10^4$/well) were then cultured for 5 days in medium containing with penicillin 100 units/ml and streptomycin 100 mg/ml at 37℃ in 5% CO_2 in air. The cells will be transfected with siRNA formulated into liposomes (LipofectAMINE™ 2000, Invitrogen) according to the manufacturer's instructions. Cells will be harvested for analysis at various times thereafter.

RNA extraction and quantitative PCR: Cells are resuspend in Trizol reagent (Life Technologies Ltd, Paisley, UK) and RNA extracted according to the manufacturer's protocol. Residual DNA is removed by treatment with 5 units of DNase I (Clontech Laboratories, Basingstoke, UK) at 37℃ for 45 min followed by inactivation at 65 ℃ for 10 min. Quantification of STAT4 mRNA is performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions. Reactions are performed in a 20 µl volume with 0.5 µM primers and $MgCl_2$ concentration optimized between 2-5 mM. Nucleotides, Taq DNA polymerase, and buffer are included in the LightCycler-RNA amplification system (Roach, Cat. No. 2015137). A typical protocol took approximately 15 min to complete and

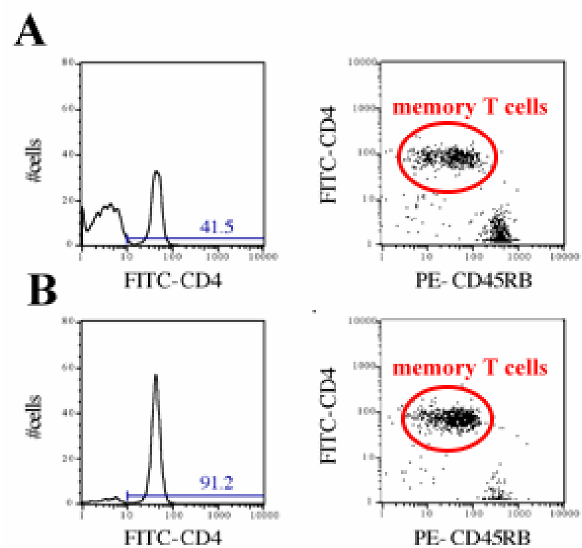

Figure 3 FACS analysis of memory $CD4^+T$ cells. A. $CD4^+T$ cells from the lymphnodes of mice. B. $CD4^+T$ cells were highly enriched by using Mouse Memory T cell $CD4^+/CD62L^-/CD44^{high}$ Column Kit (R&D).

included a 30 s denaturation step followed by 40 cycles with a 95 °C denaturation for 0 s, 55 °C annealing for 5 s, and 72 °C extension for 5 s. Extension periods varied with specific primers depending on the length of the product (~1 s/25 bp). Detection of the fluorescent product was carried out either at the end of the 72 °C extension period or after an additional 2 s step at 2 °C below the product Tm. To confirm amplification specificity the PCR products from each primer pair are subjected to a melting curve analysis and subsequent agarose gel electrophoresis. The quantification data were analyzed with the LightCycler analysis software. Normalization of samples is performed by dividing the copies of STAT4 mRNA by the copies of GAPDH reference mRNA.

Analysis of STAT proteins, ELISA: Cells were lysed in 1 ml of lysis buffer (PBS with 0.01% Triton X-100 nonionic detergent)

containing protease inhibitors. Debris-free supernatants were isolated and the cytokines or STAT proteins were measured by ELISA. Ab pairs from R&D Systems (Minneapolis, MN) were used for ELISAs. ***Immunoblotting:*** After SDS-PAGE resolution, immunoblotting was performed with anti-Stat Abs. Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20, 5% nonfat dried milk, and 1% BSA, and incubated sequentially with anti-phosphoStat3 (1/1000; New England Biolabs, Beverly, MA) and anti-Stat3 (1/1000; Santa Cruz Biotechnology), or anti-phosphoStat4 (1/1000; Zymed, San Francisco, CA) and anti-Stat4 (1/1000; Santa Cruz Biotechnology), followed by HRP-conjugated anti-rabbit IgG (1/5000).

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計畫成果自評

In this study, we had induced a transient suppression of STAT4 by transfecting $CD4^{+}/CD45RB^{low}$ memory T cells with specific anti-STAT4 siRNA. Then we had verified wheather the STAT4-silenced memory T cells response to IL-23. Similar experiments aims to examine the role of STAT1, STAT3, or STAT5 in IL-23 signal transduction had also been taken. It is found that the STAT4-silenced memory T cells would not response to IL-23. These results demonstrated that STAT4 may

play an essential role in IL-23 signal transduction.