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〔Original Paper〕

AP-1 may be a target of Th-subset-specific signaling and may regulate distinct cytokine expression patterns in Th subsets

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SUMMARY

The Th1 and Th2 subsets in murine $CD4^+$T helper cells are distinguished by their cytokine secretion patterns. To get insight into mechanisms which lead to the distinct cytokine secretion patterns in Th1 and Th2 cells, I analyzed the transcription factor, AP-1 in these cells. Using a probe containing a canonical AP-1 binding site, nuclear extracts from T cell clones were examined for EMSA. Nuclear extracts from Th1 cell clones, HDK-1 and D1.1 cells, formed two distinct bands, one fast migrating and the other slowly migrating bands. Those of Th2 cell clones, D10. G4. 1, and CDC25 cells, formed a dominant, fast migrating band. Western blot analyses of the nuclear extracts indicated that JunB and c-Fos were more abundant in nuclear extracts from Th2 cell clones than in nuclear extracts from Th1 cell clones after stimulation with PMA plus A23187. Furthermore, D10. G4. 1 exhibited a higher mRNA level of c-Fos than that of HDK-1 after the stimulation, although I did not detect a substantially different mRNA level of JunB in these cells. My results indicate that AP-1 may contribute to the distinct lymphokine secretion patterns in Th1 and Th2 cells.

Key words : Th1/Th2, Gene regulation, Signal Transductions/Cytokines, Transcription Factors

Abberviations : EMSA : electrophoretic mobility shift assay

I. Introduction

The Th1 and Th2 subsets in murine $CD4^+$T helper cells are distinguished by their cytokine secretion patterns [1]. Activated Th1 cells produce IFN-γ, lymphotoxin, and IL-2, whereas activated Th2 cells produce IL-4, IL-5, and IL-13. Each subset exerts distinct effector functions through secretions of distinct sets of cytokines. Th1 cells are responsible for cell-mediated immune responses, and Th2 cells direct allergic

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or anti-inflammatory responses. During development from naive cells to polarized subsets, several factors can contribute to polarization, such as the dose and kind of antigens, particular antigen presenting cells, costimulatory signaling pathways, and particular cytokines. Notably, IL-12 and IL-4 are pivotal factors in driving the development of Th1 cells and Th2 cells, respectively. Committed Th1 and Th2 cells are reported to be different in several respects, namely, TCR-mediated signaling pathways [2, 3], and cytokine receptor-mediated signaling pathways, including IFN-γ [4, 5], and IL-12 receptors [6]. However, exact mechanisms, which lead to the distinct cytokine secretion patterns in Th1 and Th2 cells, remain to be clarified.

To investigate the mechanisms, transcriptional regulations of IL-2 and IL-4 gene expression in Th1 and Th2 cells have been studied [7, 8]. Several transcription factors — such as NFAT, AP-1, and Oct — are known to bind to both the IL-2 promoter region and the IL-4 promoter region [8-11].

AP-1 transcription factors mediate transactivation of genes containing consensus sequence (5'-TGA C/G TCA-3'), termed TPA-responsive element (TRE) [12]. AP-1 proteins comprise the Fos and Jun families. Homodimers of Jun family members, but not of Fos family members, can bind to TRE. Binding of Fos family members to TRE requires heterodimerization with Jun family members. Heterodimers of Jun and Fos family members possess higher binding affinity and stronger transactivation activity than dimers of Jun family members. AP-1 binding sites were found in the promoter region of IL-2, IFN-γ, GM-CSF, IL-3, IL-4, and IL-5.

Therefore, I examined the possibility that AP-1 was a direct or indirect target of Th-subtype-specific signaling which leads to distinct cytokine expression patterns in Th1 and Th2 cells.

II. Materials and Methods

Cell lines

The Th1 cell clones HDK-1 and D1.1, and the Th2 cell clones D10.G4.1 and CDC25, used in this study have been described previously [13]. All clones were maintained in T media (RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, 100 U/ml penicillin G, 100 mg/ml streptomycin, 10mM HEPES, 1mM sodium pyruvate, 2mM L-glutamin, and 200 U/ml mouse rIL-2) and stimulated bimonthly with 3000 rad-irradiated synergenic spleen cells and Ags.

Fourteen days after stimulation with spleen cells and Ags, T cell clones were stimulated at 5×10^6 cell/ml in T media without mouse rIL-2 under one of the following conditions : 50 ng/ml of PMA (Calbiochem Co., La Jolla, CA) and /or 0.5 μM of A23187 (Calbiochem Co.). Protein concentration was measured by using Bio-Rad Protein Assay kit II (Bio-Rad).

EMSA

Nuclear extracts were prepared as described previously [14]. Two or 4 μg of nuclear extracts were incubated for 30 min at room temperature in a final volume of 10 μl containing 100 mM KCl, 10 mM HEPES-NaOH (pH7.9), 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 50 μg/ml poly (dI-dC) (Pharmacia Biotech Inc, Piscataway, NJ), 250 μg/ml bovine serum albumin, and approximately 0.5 ng of labeled probe (approximately 50,000 cpm). The resulting DNA-protein complexes were separated by electrophoresis on a 0.25 x TBE/4% non-denaturing gel at room temperature. For super-shift analyses, the reaction mixtures were incubated in the presence of antibodies for 2 h on ice before the addition of labeled probes. The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) : c-jun/AP-1 (D) (sc-44 X), and c-fos (K-25) (sc-253 X)

Sequences of synthetic oligonucleotides used

were as follows (5' overhangs are presented in lower case, and only one strand is shown) : canonical AP-1 5'-tcgaGCTATGACTCATCCG-3' [15]

Western blot analysis

Equal amounts of nuclear extract protein mentioned in figure legends, as determined by Bio-Rad Protein Assay kit II® (Bio-Rad), were loaded into each lane of 7.5% SDS-PAGE gels and electrophoretically separated. Proteins were electrophoretically transferred onto Immobilon-P® membranes (Millipore Corp., Bedford, MA). Membranes were preincubated for 1 h at room temperature in PBS, pH 7.6, containing 0.5% Tween-20 (Sigma, ST. Lous, Mo) (TPBS) and 3.75% Bovine Albumin Frac-tion V (Gibco BRL., Gaithersburg, MD). The membranes were incubated in primary antibodies at 0.5 μg/ml in 3.75% Bovine Albumin Fraction V-TPBS for peroxydase-labeled donkey anti-rabbit antisera (Amersham International, Buckinghamshire. UK) diluted 1 : 10,000 in TPBS. Blots were then developed using the ECL® detection kit (Amersham International). The following antibodies were used : c-jun AP-1 (N) (sc-45), jun B (N-17) (sc-46), jun D (329) (sc-74), c-fos (4) (sc-52), fos B (102) (sc-48) (Santa Cruz Biotechnology, Inc.).

Northern blot analysis

Poly (A)+RNAs were prepared by using Fast Track® 2.0 kits (Invitrogen, San Diego, CA). For hybridization probes of JunB and c-Fos, I used internal *Sal* I fragments of pRSVJunB and pRSV-c-Fos, respectively. For β-actin, I used the 540 bp fragment which corresponds to position 183 to 722 in murine β-actin. Samples of Poly (A)+RNAs (0.5 μg/lane) were size-fractionated by electrophoresis on 1.2% agarose, 2.2 M formaldehyde gels, and then blotted onto Nytran Plus® (Schleicher & Schuell, Keene, NH). Hybridization was performed with ^{32}P-labeled specific DNA probes labeled by Prime-it® II (Stratagene, La Jolla, CA) at 68℃ for 2 h. Filters were washed with 0.1% SDS-2 x SSC at room temperature twice for 15 min each and with 0.1% SDS-0.1 x SSC at 60℃ for 30 min, and were autoradiographed by using Biomax® films (Eastman Kodak, Rochester, NY) and intensifying screens at-70℃.

Ⅲ. Results

Different complex formations of nucleoproteins from Th1 and Th2 cells with AP-1 oligonucleotides

To analyze AP-1 proteins in Th subsets, I performed EMSA using oligonucleotides containing a canonical AP-1 binding site, since the proximal AP-1 site is a relatively low affinity site for AP-1 bindings [16] (Fig. 1A). Two Th1 cell clones, HDK-1 and D1.1, and two Th2 cell clones, D10.G4.1 and CDC25 were stimulated with PMA and/or A23187 for 2 hours before preparation of nuclear extracts. Nuclear extracts from Th1 cell clones formed two distinct bands. The two bands were clearly observed with or without the stimulation of PMA and/or A23187. On the contrary, nuclear extracts from Th2 cells a formed a strong, fast migrating band and a weak, slowly migrating band. Especially, nuclear extracts form D10.G 4.1 gave a barely detectable, slowly migrating band. Nuclear extracts from unstimulated CDC25 formed the slowly migrating band which was only weakly visible, and PMA or PMA plus A23187 strongly enhanced the fast migrating band, while the intensity of the slowly migrating band did not change or rather decreased compared with the band observed without the stimulation. These bands observed in Th1 and Th2 cells were further characterized by super-shift analyses using antibodies against Jun and Fos in HDK-1 and D10.G4.1 stimulated with PMA plus A23187 for 2 hours (Fig. 1B). The antibody, c-jun/AP1 (D), which is broadly reactive with the 39 kd c-jun, junB, and junD

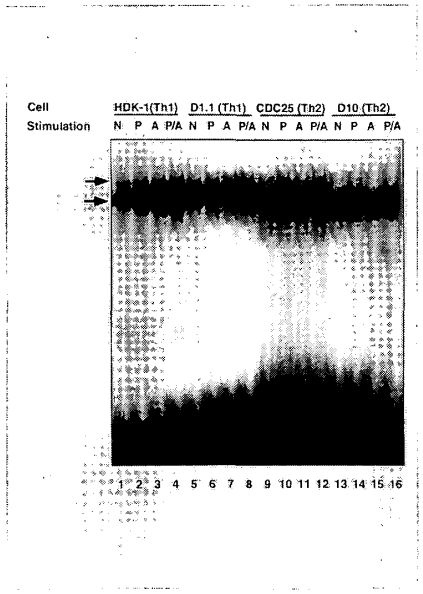

Fig. 1A

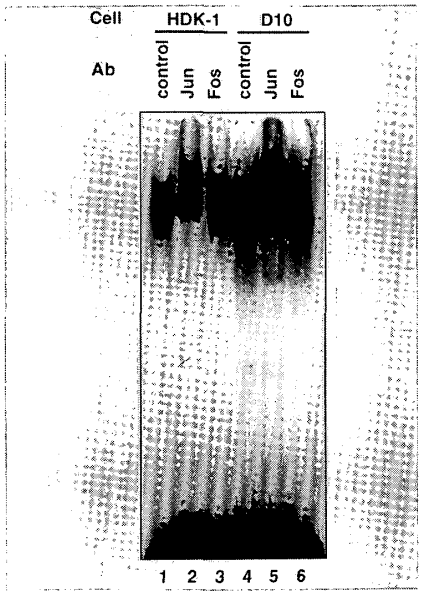

Fig. 1B

Comparison of bindings to canonical AP-1 site in Th subsets. A, T cell clones were unstimulated (N), or stimulated with 50 ng/ml of PMA (P), and/or 0.5 μM of A23187 (A) for 2 h ; and EMSAs were performed with nuclear extracts (2 μg/lane). B, supershift analyses were performed with nuclear extracts (4 μg/lane) from HDK-1 and D10. G4. 1 cells stimulated with PMA plus A23187 for 2 hours. 2 μg of antibodies were added to reaction mixtures.

encoded proteins, partially abolished both the fast and the slowly migrating bands. On the other hand, the antibody, c-fos (K-25), which reacts with c-Fos, FosB, Fra-1, and Fra-2, partially abolished the fast migrating band, but not the slowly migrating band. In addition, recombinant human c-Jun formed one band whose mobility was the same as the mobility of the slowly migrating band (data not shown). Therefore, I take this to mean that the fast migrating band contains heterodimers of Jun and Fos family members, and that the slowly migrating band contains homodimers of Jun family members.

These observations strongly suggest that nuclear extracts from Th1 cells bind to the AP-1 site as both heterodimer of Jun and Fos family members and homodimers of Jun family members, although nuclear extracts from Th2 cells bind to this site mostly as heterodimers of Jun and Fos family members.

JunB and c-Fos protein were more abundant in nuclear extracts from Th2 cell clones than in nuclear extracts from Th1 cell clones

To examine the different bindings of nuclear extracts from Th1 and Th2 cell clones to the AP-1 binding site, I analyzed the composition of AP-1 proteins in nuclear extracts from HDK-1 and D10. G4.1 by Western blot analyses (Fig. 2A). Stimulation with PMA plus A23187 for 2 hours increased amounts of c-Jun, JunB, and c-Fos in nuclear extracts from both HDK-1 and D10. G4. 1, while amounts of JunD and FosB did not change. Nuclear extracts from HDK-1 and D10. G4. 1 stimulated with PMA plus A23187 contained similar amounts of c-Jun, JunD, and FosB proteins. However, nuclear extracts from D10. G4. 1 contained obviously more JunB and c-Fos proteins than those contained in nuclear extracts from HDK-1. Titrations of each nuclear extracts from these cells stimulated with PMA plus A23187 indicated that nuclear extracts from D10. G4. 1 contained 2-to 4- fold more both JunB and c-Fos proteins, compared with those from HDK-1 (Fig. 2B). The abundance of both JunB and c-Fos in nuclear extracts from Th2 cell clones, compared with

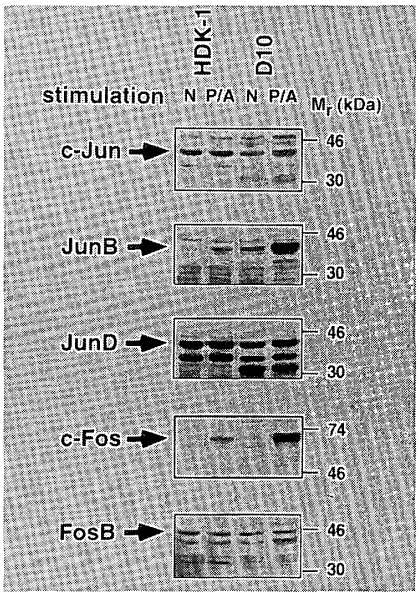

Fig. 2A

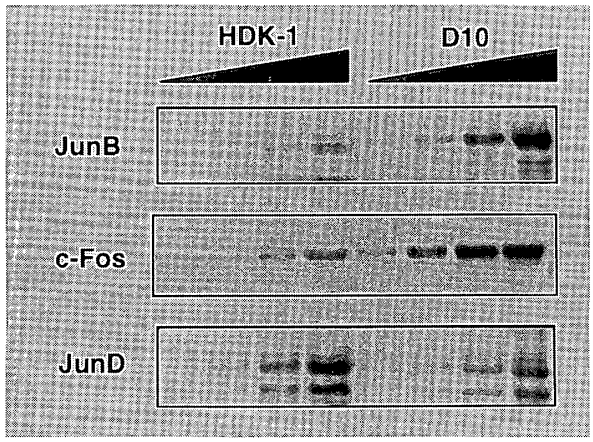

Fig. 2B

Western blot analysis of Jun and Fos family members in Th subsets. A, HDK-1 (Th1) and D10. G4.1(Th2) were unstimulated or stimulated with PMA (50 ng/ml) plus A23187 (0.5 μM) for 2 h. Nuclear extracts were prepared, separated by SDS-PAGE (30 μg/lane), and transferred to PVDF membranes. The blots were probed with antibodies specific for each Jun and Fos family member and horseradish peroxydase-conjugated donkey anti-rabbit antisera, and developed using the ECL detection kit. B, titrations were performed by applying different amounts of nuclear extracts from HDK-1 and D10. G4.1 stimulated with PMA (50 ng/ml) plus A23187 (0.5 μM) for 2 h. 3.8 μg (lanes 1 and 5), 7.5 μg (lanes 2 and 6), 15 μg (lanes 3 and 7), and 30 μg (lanes 4 and 8) of nuclear extracts were applied.

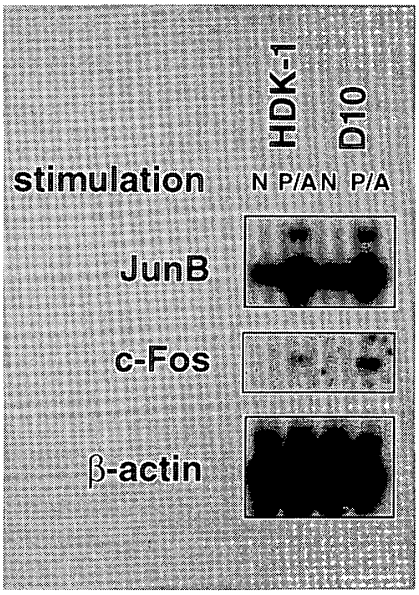

Fig 3. Northern blot analyses of JunB and c-Fos in Th subsets. HDK-1 (Th1) and D10. G4.1 (Th2) were unstimulated or stimulated with PMA (50 ng/ml) plus A23187 (0.5 μM) for 1 h. Poly (A)$^+$RNAs (0.5 μg) were separated by formaldehyde-agarose gel electrophoresis and transferred to nylon membranes. The blots were probed with a ^{32}P-labeled cDNA probe specific for junB, c-fos, or β-actin. Radioactive bands were visualized by 3 day autoradiography at -70℃.

those from Th1 cell clones after PMA plus A23187 stimulation for 2 hours, were also observed from the comparison of nuclear extracts from D1.1 (Th1) and CDC25 (Th2), although the difference in the amount of JunB protein was less prominent than the difference in nuclear extracts from HDK-1 and D10. G4.1 (data not shown).

Northern blot analyses were performed on HDK-1 and D10. G4.1 stimulated with or without PMA plus A23187 for 1 h (Fig. 3). Induction of both JunB and c-Fos mRNA was observed on both of these cells. However, D10. G4.1 exhibited a higher mRNA level of c-Fos than that of HDK-1 after the stimulation, although I did not detect a substantially different mRNA level of JunB in these cells.

Ⅳ. Discussion

To investigate mechanisms, which lead to the distinct cytokine secretion patterns in Th1 and Th2 cells, I attempted to evaluate differences

of AP-1 in Th1 and Th2 cells, since several AP-1 binding sites exist in the promoter region of the IL-2 gene, which can be induced in Th-subset-specific manner. EMSA of nuclear extracts from Th1 and Th2 cells displayed the different binding complexes formed with the canonical AP-1 binding site (Fig. 1A). Stimulation of PMA plus A23187 induced more JunB and more c-Fos in nuclear extracts from Th2 cell clones than those from Th1 cell clones (Fig. 2).

I do not know the mechanism of more abundant nuclear accumulation of JunB in Th2 cells than in Th1 cells after the stimulation. HDK-1 and D10. G4.1 did not show a significant difference in the level of mRNA expression of JunB (Fig. 3). Different subcellular localization of JunB in these cells is unlikely, because the result of Western blot analysis of JunB on whole cell extracts was consistent with that on nuclear extracts (data not shown). Other possibilities may be regulations at the level of translation and/or turnover of JunB protein [17].

Abundance of c-Fos in Th2 cell clones after the stimulation was also observed at the level of mRNA as well as the level of protein (Fig. 3). Several *cis* elements of the c-fos gene mediate c-Fos induction in response to a diverse spectrum of extracellular stimuli [12, 18]. These elements inclued cAMP response element (CRE), serum response element (SRE), and sis-inducible enhancer (SIE), and may determine the difference in the level of mRNA of c-Fos in Th1 and Th2 cell clones.

The different amounts of JunB and/or c-Fos in nuclear extracts from Th1 and Th2 cells may explain the differences of several Th-subset-specific gene expression in these cells. Different transactivation activities of Jun family members have been reported. Comparison of c-Jun and JunB showed that c-Jun is an efficient transactivator for AP-1 dependent promoters, but JunB is rather ineffective, and that JunB represses transactivation and transformation induced by c-Jun [19-21]. Difference in transactivation activity of Jun family members was also confirmed in the heterodimer with c-Fos, and among them JunB-c-Fos heterodimer was least efficient [22]. The decreased transactivation activity of JunB was reported to result from its decreased DNA binding activity and from its unstable leucine zipper [23].

However, more abundant c-Fos in nuclei of D10. G4.1. than in those of HDK-1 seems contradictory to Th1-specific IL-2 gene expression, since transfection of a Th1 cell clone with c-Fos expression plasmids has been shown to induce increased IL-2 promoter activity [24], and splenic T cells from c-Fos transgenic mice have dis-played increased and sustained IL-2 production than T cells from control mice [25], although spleen cells from c-Fos deficient mice have shown similar levels of IL-2 productions [26]. One possible reason for this discrepancy may be because other mechanisms such as post-transcriptional regulation of c-Fos may be different in Th1 and Th2 cells [27] and decisive for the regulation of IL-2 gene in these cells.

Jun and Fos family members can form complexes with some of ATF/CREB family members and bind to CRE and/or AP-1 sites as heterodimers [28]. In addition, Jun and Fos family members can also form heterodimers with Maf/Nrl family proteins and bind to Maf recognition elements (MAREs) [29]. These suggest that the regulation of transactivation activity by the difference in the composition of AP-1 may also function through CRE and/or MAREs.

In summary, I identified that AP-1 is different in Th1 and Th2 cells, and this indicate that AP-1 may be a target for the distinct lymphokine secretion patterns in Th1 and Th2 cells, although upstream events leading to different compositions of AP-1 proteins and downstream events caused by them remain to be clarified.

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要　旨

マウスCD4陽性ヘルパーT細胞は産生するサイトカインの違いによりTh1とTh2サブセットに分類される。Th1とTh2細胞における異なるサイトカイン産生の機構を明らかにするため，これら細胞における転写因子AP-1について検討した。標準的AP-1結合部位を含有するプローブを用いて，T細胞クローンの核抽出液をゲルシフト法にて解析した。Th1細胞クローンのHDK-1とD1.1細胞は移動度の異なる明瞭な2本のバンドを呈した。Th2細胞クローンのD10. G4.1とCDC25細胞では早い移動度を呈するバンドに一致するバンドのみを明らかに認めた。各種抗Jun／Fos抗体によるスーパーシフトにより，早い移動度と遅い移動度のバンドは各々Jun／Fos, Jun／Junファミリーの複合体であると示唆された。PMAとA23187で刺激したTh2細胞クローンではTh1細胞クローンよりも核抽出液中のJunBとc-Fosが多いことをウェスタンブロッティング法で示した。また上記刺激によりD10. G4.1ではHDK-1よりもc-FosのmRNA発現の増加をみとめた。以上の結果からTh1とTh2細胞におけるAP-1の構成成分の違いが，これらの細胞における異なるサイトカイン産生機構に関与している可能性が示唆された。

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