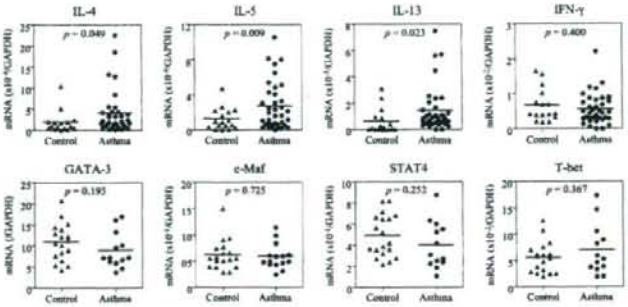
Figure No.1

Fig. 1.



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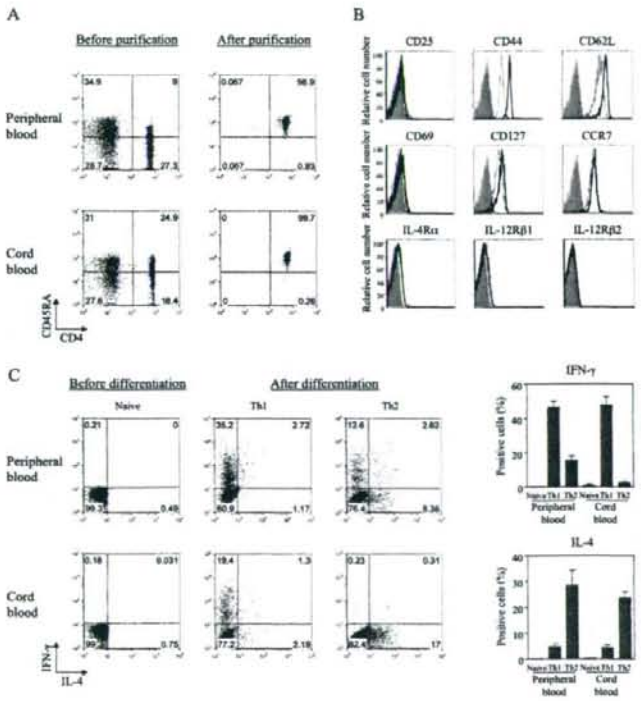

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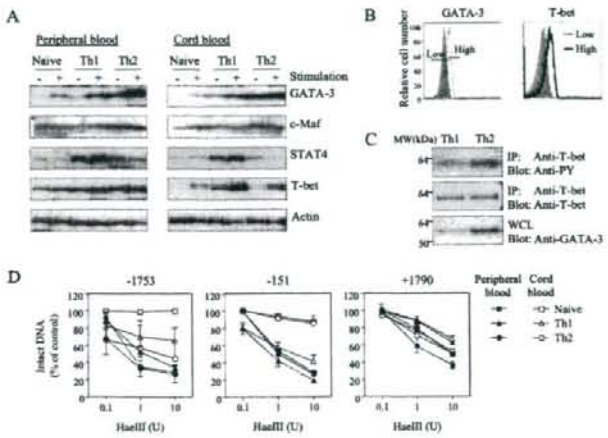

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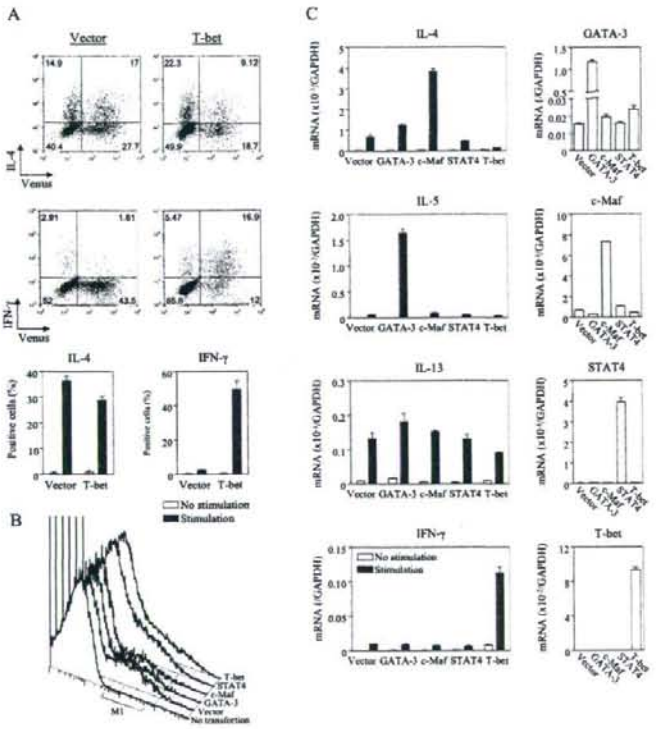

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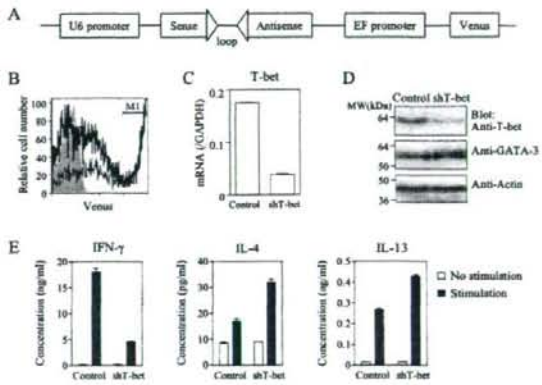

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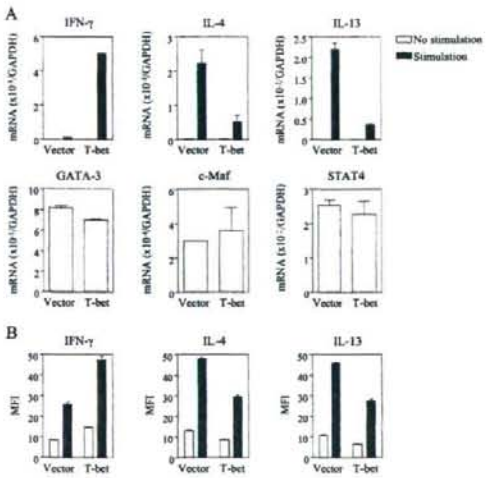
Fig. 6.



ONLINE REPOSITORY

T-bet is responsible for distorted Th2 differentiation in human peripheral CD4$^+$ T cells

Osamu Kaminuma DVM, PhD[a]*, Fujiko Kitamura BSc[a], Shoichiro Miyatake MD, PhD[b], Kazuko Yamaoka PhD[a], Hiroyuki Miyoshi PhD[c], Shigeko Inokuma MD, PhD[d], Hideki Tatsumi, MD[e], Soichi Nemoto MD[e], Noriko Kitamura MSc[f], Akio Mori MD, PhD[f] and Takachika Hiroi DDS, PhD[a]

[a]Department of Allergy and Immunology and [b]Cytokine Project, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan

[c]Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN, Tsukuba Institute, Ibaraki 305-0074, Japan

[d]Department of Allergy and Rheumatology, Tokyo Metropolitan Komagome Hospital, Bunkyo-ku, Tokyo 113-8613, Japan

[e]Department of Obstetrics and Gynecology, and [f]Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Sagamihara, Kanagawa 228-8522, Japan

FIG.E1. mRNA expression of GATA-3 and T-bet in peripheral and cord blood CD4+ T cells. Purified naïve CD4+ T cells and differentiated Th1 and Th2 cells from peripheral and cord blood of healthy subjects was determined by quantitative real-time RT-PCR method. Data are expressed as means ± SEM as mRNA abundance normalized with GAPDH expression (n = 3-6).

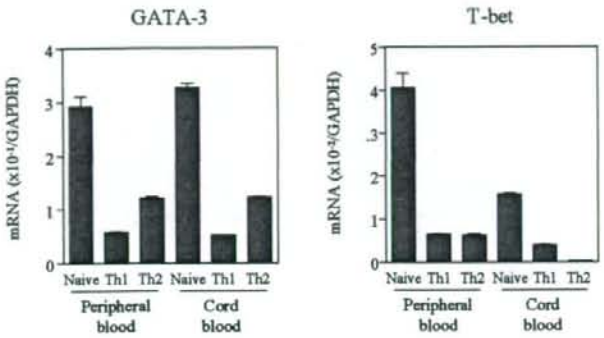


FIG.E1. mRNA expression of GATA-3 and T-bet in peripheral and cord blood CD4+ T cells. Purified naïve CD4+ T cells and differentiated Th1 and Th2 cells from peripheral and cord blood of healthy subjects was determined by quantitative real-time RT-PCR method. Data are expressed as means ± SEM as mRNA abundance normalized with GAPDH expression (n = 3-6).

TABLE E1. Probes used for quantitative real-time RT-PCR.

Probe	Sequence
IL-4	GGCGGGCTTGAATTCCTGTCCTGTG
IL-5	ATAAAAATCACCAACTGTGCACTGA
IL-13	GAACCAGAAGGCTCCGCTCTGCAAT
IFN-g	GAAATATTTTAATGCAGGTCATTCA
GATA-3	GACCCACCACGGGAGCCAGGTGTGC
c-Maf	GTTTTTCATAACTGAGCCCACTCGC
STAT4	GTAAAGGAAATGAGGGCTGTCACAT
T-bet	CAAGCAGGGACGGCGGATGTTCCCA

TABLE E2. 5'- and 3'- primers and specific probe sets used to detect the digestion of the T-bet gene in REA assay.

HaeIII site		Sequence
-1753	5' primer	-1792 GGAGAATGGTGGGTAAGGTGTTG -1769
	3' primer	-1726 CGGATACATGACAGTACCAGAAACA -1750
	Probe	-1751 CAGGACTGGCCTCCTC -1766
-151	5' primer	-183 GCAGGCTCTCGCTTCTCT -166
	3' primer	-121 CAAATTCTACGCTCTTGGCGAGTA -143
	Probe	-145 CTGCAGGGCCCCCCAT -160
+1790:	5' primer	+1767 TGCGCACCCACCCTTC +1782
	3' primer	+1824 CGCTGGTCCCCTGCT +1810
	Probe	+1784 CAGTGCGGCCCATGTG +1809

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Downregulation of IL-13 Gene Transcription by T-bet in Human T Cells

Kazuya Suzuki[a, c] Osamu Kaminuma[a] Takachika Hiroi[a] Fujiko Kitamura[a]
Shoichiro Miyatake[b] Fumio Takaiwa[c] Hideki Tatsumi[d] Soichi Nemoto[d]
Noriko Kitamura[e] Akio Mori[e]

[a]Department of Allergy and Immunology and [b]Cytokine Project, Tokyo Metropolitan Institute of Medical Science, Tokyo,
[c]Transgenic Crop Research and Development Center, National Institute of Agrobiological Sciences, Tsukuba,
[d]Department of Obstetrics and Gynecology and [e]Clinical Research Center for Allergy and Rheumatology,
National Hospital Organization, Sagamihara National Hospital, Sagamihara, Japan

Key Words
IL-13 · T-bet · T cell · Th1/Th2

Abstract
Background: Downregulation of a Th2 cytokine, IL-4, by a Th1-specific transcription factor, T-bet, has been demonstrated. However, the regulatory role of T-bet in another Th2 cytokine, IL-13, is not fully delineated. ***Methods:*** IL-13 mRNA expression in Jurkat cells was examined by quantitative RT-PCR, while the transcriptional activity of 5′-flanking region in the IL-13 gene encompassing –1077 to +49 was investigated by fluorescence-based promoter reporter assay. The effect of T-bet was investigated by transfection of the cells with the T-bet expression vector. ***Results:*** Stimulation with phorbol ester plus Ca^{2+} ionophore clearly induced IL-13 gene transcription in Jurkat cells. Ectopically expressed T-bet significantly suppressed the inducible mRNA expression and promoter activity of IL-13. ***Conclusion:*** IL-13 expression was downregulated by T-bet at the level of gene transcription, independently of the modulation of Th1/Th2 balance. T-bet is the potential key factor in the development of Th1/Th2-related diseases.

Introduction

Naive CD4+ T cells differentiate into 2 functional subsets, Th1 and Th2, on the basis of the cytokine production pattern [1]. Th1 cells specifically produce IFN-γ, which controls cellular immunity, while Th2 cells preferentially secrete IL-4, IL-5 and IL-13, which are involved in the regulation of humoral immunity [2, 3]. Impairment of Th1/Th2 balance has been suggested to cause variety of diseases. For example, acceleration of Th1 response is recognized in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, whereas excessive Th2 responses are observed in allergic diseases including bronchial asthma and atopic dermatitis [2, 3].

Th1 differentiation is crucially regulated by a transcription factor, T-bet. The expression of T-bet in T cells is correlated with the polarization of Th1 phenotype [4]. Ectopically expressed T-bet induced IFN-γ synthesis in Th2-polarized cells [4, 5]. T cells derived from T-bet-deficient mice were defect in Th1 differentiation [6] and, accordingly, these mice spontaneously suffered from Th2-related disorders [7]. These data suggest that T-bet plays a key role in the development of Th1/Th2 unbalance-related multiple diseases. Not only augmentation of Th1 cytokine synthesis, but also downregulation of a Th2

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Correspondence to: Dr. Akio Mori
National Hospital Organization, Sagamihara National Hospital
Clinical Research Center for Allergy and Rheumatology
18-1 Sakuradai, Sagamihara, Kanagawa 228-8522 (Japan)
Tel. +81 42 742 8311, Fax +81 42 742 7990, E-Mail mori-kkr@umin.ac.jp

cytokine is induced by T-bet. Thus, we have recently demonstrated that IL-4 gene transcription in human T cells was suppressed by T-bet [8]. However, the direct contribution of T-bet to the synthesis of another Th2 cytokine, IL-13, has not been elucidated. In this study, we investigated the effect of T-bet on mRNA expression as well as the promoter activity of IL-13 by employing human T cell line.

Material and Methods

Plasmids

Human T-bet cDNA amplified from total cDNA of human peripheral blood lymphocytes was subcloned into the pEF6/His expression vector (pEF-T-bet; Invitrogen, Carlsbad, Calif., USA). The human IL-13 promoter (–1077 to +49) was subcloned into the pEGFP-1 vector (IL-13-EGFP; BD Bioscience, Palo Alto, Calif., USA). The correct sequences of all constructs were verified by sequencing.

Measurement of IL-13 mRNA in Human T Cells

Jurkat cells were transfected with pEF-T-bet or empty vector as described [8]. At 48 h after transfection, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin for 6 h at 37°C. Total RNA was then extracted and reverse transcribed using oligo(dT) 12–18 primer and ReverTra ACE (Toyobo, Osaka, Japan). Quantitative real-time RT-PCR was performed using Assays-on-Demand™ Gene Expression Products with an ABI prism 7900 sequence detection system (Applied Biosystems, Foster City, Calif., USA). IL-13 transcripts were normalized to GAPDH abundance.

Reporter Assay

After 16 h of stimulation, fluorescence of synthetic EGFP in the transfected cells was determined by flow cytometry. Quantitative data are expressed as mean fluorescence intensity as described [9].

Statistical Analysis

Statistical analysis was performed with Student's t test, and $p < 0.05$ was considered statistically significant.

Results and Discussion

T-bet is strongly implicated in the commitment to Th1 phenotype, mainly due to its promoting activity for IFN-γ gene transcription [4]. On the other hand, we have recently demonstrated that IL-4 gene transcription was also suppressed by T-bet [8]. However, whether T-bet directly affects IL-13 is unclear. To elucidate how T-bet suppresses Th2 reactions, we examined the effect of T-bet on mRNA expression and the promoter activity of IL-13 in human T cells employing quantitative real-time RT-PCR and fluorescence-based promoter reporter assay. As shown in table 1, IL-13 mRNA expression in Jurkat cells was clearly augmented by stimulation with PMA plus ionomycin. Accordingly, the transcriptional activity of an approximately 1-kb fragment of the IL-13 promoter was enhanced upon stimulation, consistent with previous investigations [10]. The inducible expression of IL-13 mRNA as well as its promoter activity in Jurkat cells was suppressed by ectopic expression of T-bet (table 1).

Table 1. Effect of T-bet on mRNA expression and the promoter activity of IL-13 in human T cells

Stimulation	mRNA, $\times 10^{-3}$/GAPDH		Promoter activity, MFI	
	–	+	–	+
Vector	0.000 ± 0.000	1.083 ± 0.026[b]	15.6 ± 0.7	38.4 ± 0.9[b]
T-bet	0.000 ± 0.000	0.067 ± 0.002[b, d]	11.7 ± 2.3	21.6 ± 3.0[a, c]

Jurkat cells were transfected with pEF-T-bet or empty vector in the presence or absence of IL-13-EGFP (10 μg each). After 48 h, cells were either left unstimulated or stimulated with PMA (5 nM) plus ionomycin (1 μM) for 6 h and the expression of IL-13 mRNA was determined by quantitative real-time RT-PCR. Data are expressed as means ± SEM of mRNA abundance normalized with GAPDH expression (n = 4). At 24 h after transfection, reporter vector-transfected cells were stimulated for 16 h and the promoter activity was detected as the fluorescence of synthetic EGFP measured by flow cytometry (n = 4). MFI = Mean fluorescence intensity.

[a] $p < 0.05$; [b] $p < 0.01$ compared with nonstimulated control. [c] $p < 0.05$; [d] $p < 0.01$ compared with empty vector-transfected control.

In accordance with our present findings, it has been demonstrated that IL-13 production by CD4+ T cells was upregulated in T-bet-deficient mice [11]. However, the mechanisms by which T-bet diminishes IL-13 expression are unclear. Even though Th1 and Th2 cytokines counteract each other, downregulation of IL-13 by T-bet (table 1) was not likely to be achieved through the enhancement of IFN-γ expression, since Jurkat cells failed to produce the detectable amount of IFN-γ in the culture supernatant (determined by ELISA; data not shown). We have demonstrated that T-bet played a negative role in IL-13 synthesis during the development of Th2 subset from human naive CD4+ T cells (data not shown). Therefore, further investigation will be required into the direct effect of T-bet on IL-13 gene transcription in human peripheral T cells.

It is intriguing that the sequence ACACCATGGTCT, located at –52 to –63 in the human IL-13 promoter is homologous to the consensus T-box-binding site

(ACACxxxxGTGT). Although the functional significance as well as T-bet-binding activity have not been clarified, this region is contiguous to the consensus GATA-binding site (AGATAG) at –79 to –85. It has been demonstrated that GATA-3 associated with this GATA site crucially contributes to IL-13 gene transcription in T cells [12]. Hwang et al. [13] demonstrated that T-bet directly interacted with GATA-3 and sequestered it away from binding to target DNA. In addition, suppression of GATA-3 expression by T-bet in murine T cells has been suggested [14]. Therefore, there is a possibility that T-bet-mediated inhibition of IL-13 transcription was not a direct effect on the IL-13 promoter, but due to the repression of GATA-3. Further examination will be required to elucidate the role of T-bet as well as the T-box element in the IL-13 promoter in GATA-3 expression and its activity in our experimental system.

In conclusion, IL-13 gene transcription in human T cells is suppressed by T-bet. Th1 differentiation mediated by T-bet is due not only to the transactivation of the IFN-γ gene, but also to the downregulation of Th2 cytokines. T-bet is a promising target for the treatment of Th1/Th2-related diseases.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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Differential Contribution of NFATc2 and NFATc1 to TNF-α Gene Expression in T Cells[1]

Osamu Kaminuma,[2]*† Fujiko Kitamura,*‡ Noriko Kitamura,* Takachika Hiroi,† Hiroyuki Miyoshi,§ Atsushi Miyawaki,¶ and Shoichiro Miyatake*

The NFAT family transcription factors play crucial roles in immunological and other biological events; however, the functional differences among NFAT members have not been fully elucidated. This study investigated the relative contribution of NFATc2 and NFATc1 to the transactivation of cytokine genes in T cells. Ectopic expression of NFATc2 but not NFATc1, especially its short isoform, enhanced TNF-α synthesis in human T cells at the gene transcription level, whereas both NFATs augmented IL-2 expression. In addition, a reduction of the shortest NFATc1 isoform using RNA interference technology failed to suppress TNF-α expression. The promoter/enhancer activity of the NFAT-binding site in the TNF-α gene was up-regulated by NFATc2 but not by NFATc1, whereas both NFATs associated similarly with this region. A study of mRNA expression using NFATc2/NFATc1 chimeric molecules revealed that the enhancing activity of NFAT on the TNF-α gene was lost by truncation of its C-terminal transactivation domain. In addition, this domain derived from NFATc2 behaved as a dominant negative against the NFAT site in TNF-α promoter-dependent transcriptional activity in T cells. We conclude that the C-terminal transactivation domain in NFAT is crucial for TNF-α gene expression in human T cells. ***The Journal of Immunology*****, 2008, 180: 319–326.**

The NFAT family of transcription factors consists of five members, NFATc1 to NFATc5, which are involved in the inducible expression of numerous genes concerned with immune responses as well as other biological events (1–3). NFATc1–c4 are dephosphorylated by a Ca^{2+}-dependent serine/threonine phosphatase, calcineurin, and translocate into the nucleus where they associate with target DNA sequences. The immunosuppressive drugs FK506 and cyclosporin A depress the function of these NFATs to the same degree through the inhibition of calcineurin activity (4–6).

Studies using gene-targeted mice have suggested that each NFAT family member has a differential role in the synthesis of multiple cytokines. For example, IL-4 production by T cells is impaired in NFATc1 (NFATc, NFAT2)-deficient mice (7, 8), although IL-2 synthesis is relatively enhanced (7). Nevertheless, the representative phenotypes observed in NFATc2 (NFATp, NFAT1)-deficient mice are controversial. In the first report regarding NFATc2$^{-/-}$ mice by Hodge et al. (9), a striking defect in the early production of IL-4, IL-13, GM-CSF, and TNF-α by T cells in anti-CD3 Ab-treated mice was observed in vivo, whereas IL-2 and IFN-γ were minimally affected. However, Th2 development in these mice was enhanced at later time points along with increased IL-4 production both in vitro and in vivo. Up-regulation and contrary down-regulation of Th2 cytokines in NFATc2-deficient mice were also reported later by different groups (10–13). These findings suggest at least a functional difference between NFATc2 and NFATc1 in the regulation of T cell cytokines.

However, the molecular mechanisms by which each NFAT family member plays a distinct role in cytokine synthesis still remain unclear. NFAT is composed of several functional domains (1). The DNA binding domain (DBD),[3] which lies between amino acid residues ~400 and ~700, is highly conserved within the NFAT family and shows similarity to the DBD of the Rel family proteins (14). The Ca^{2+} regulatory domain (CRD) of ~300 amino acids, which binds and is dephosphorylated by calcineurin, is located just N-terminal to the DBD. The CRD shows a lesser degree of pairwise sequence identity but strong conservation of several sequence motifs characteristic of the NFAT family (1). Both the N- and C-terminal ends of NFAT proteins contain a transactivation domain (TAD1 and TAD2, respectively) despite very limited sequence conservation (1). Amino acid homology between NFATc2 and NFATc1 is 23, 33, and 70% for TAD1, CRD, and DBD, respectively.

NFATc1 has multiple isoforms. The original form of NFATc1 was identified from a cDNA library of human peripheral blood lymphocytes and Jurkat cells (14). Thereafter, Park et al. (15) isolated a new NFATc1 isoform, NFATc.β, from a cDNA clone of the Raji B cell line. NFATc.β differs from NFATc.α (identical with the original NFATc1 that we mainly used in this study) in the first 29 N-terminal amino acid residues and contains an additional region of 142 residues at the C terminus. In addition, Lyakh et al.

*Cytokine Project, †Department of Allergy and Immunology, ‡Calpain Project, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan; §Subteam for Manipulation of Cell Fate, BioResource Center, Institute of Physical and Chemical Research, Tsukuba Institute, Ibaraki, Japan; and ¶Laboratory for Cell Function and Dynamics, Advanced Technology Development Center, Brain Science Institute, Institute of Physical and Chemical Research, Saitama (RIKEN), Japan

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[2] Address correspondence and reprint requests to Dr. Osamu Kaminuma, Department of Allergy and Immunology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan. E-mail address: kaminuma@rinshoken.or.jp

[3] Abbreviations used in this paper: DBD, DNA-binding domain; ChIP, chromatin immunoprecipitation; CRD, Ca^{2+} regulatory domain; EGFP, enhanced GFP; IRES, internal ribosomal entry site; RNAi, RNA interference; TAD, transactivation domain.

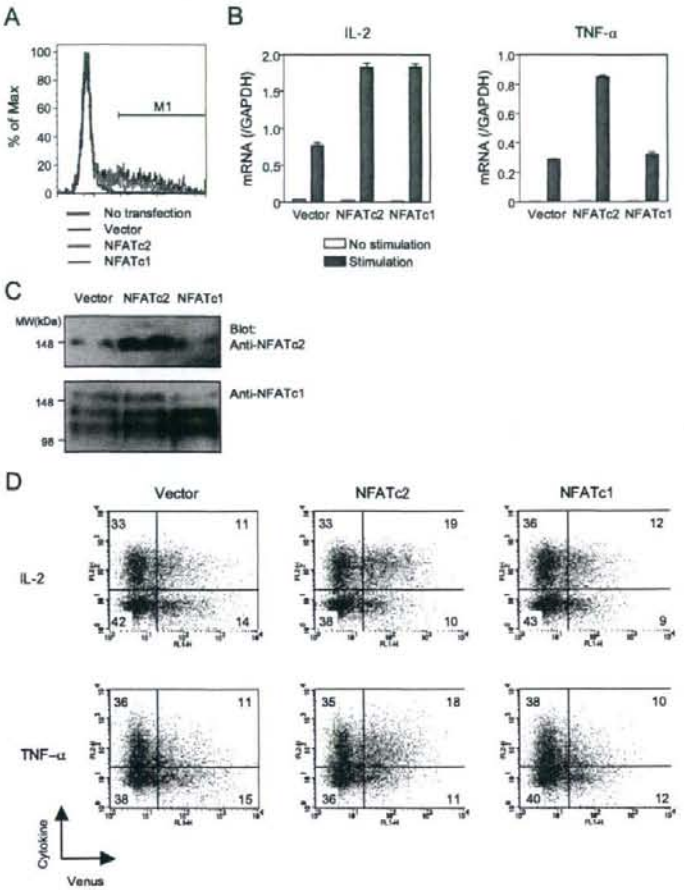

FIGURE 1. Effects of NFATc2 and NFATc1 on cytokine synthesis by T cells. Human naive or total $CD4^+$ T cells were transfected with NFATc2- or NFATc1-IRES-Venus or their empty vector (5 μg). At 48 h after transfection, transfection-positive naive $CD4^+$ T cells (M1 population) (*A*) were purified and stimulated with 2.5 μg/ml anti-CD3 Ab plus 1 μg/ml anti-CD28 Ab for 6 h, and the expression of IL-2 and TNF-α mRNA was measured by real-time quantitative PCR (*B*). Data are expressed as mean ± SEM of mRNA abundance normalized to GAPDH expression ($n = 4$). The expression of NFATc2 and NFATc1 was analyzed by immunoblotting of whole cell lysates with anti-NFATc2 and NFATc1 Abs (*C*). For intracellular staining, transfected $CD4^+$ T cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h and stained for IL-2 or TNF-α (*D*). The results shown are representative of four separate experiments.

(16) identified two short forms of NFATc1 (82 and 86 kDa), which were strongly induced by stimulation, as well as two longer and relatively consistent isoforms (110 and 140 kDa). These two short isoforms resulted from the initiation of translation at two different AUG codons (16) and were predominantly induced upon cellular activation (16, 17). In addition, Chuvpilo et al. (17) reported that the longer NFATc1 isoforms were derived by alternative polyadenylation events. However, the physiological meaning of this heterogeneity of NFATc1 is unclear.

All NFAT members expressed in T cells can activate the IL-2 promoter (18), whereas their differential effects on the NFAT binding site in the TNF-α promoter (NFAT-TNF-α) have been reported (15, 18, 19). In addition, Wu et al. (20) recently demonstrated that promoter/enhancer activity of the NFAT-binding site in the IL-2 promoter (NFAT-IL-2), but not that of NFAT-TNF-α, was suppressed by FOXP3. Therefore, to investigate the molecular mechanisms underlying the differences among NFAT family members in activating distinct cytokines, in this study we comparatively examined the effects of NFATc2 and NFATc1 on the synthesis of IL-2 and TNF-α in human peripheral T cells as well as a Jurkat T cell line by using overexpression and knockdown systems as well as NFATc2/NFATc1 chimeric molecules. The results demonstrated a crucial role of the NFATc2-TAD2 domain in activation of the TNF-α promoter.

Materials and Methods

Reagents

For the application of RNA interference (RNAi) technology, double-strand Stealth RNAi oligos (Invitrogen Life Technologies) designed using RNAi designer software (Invitrogen Life Technologies) were synthesized by Invitrogen Life Technologies. The target sequences used are as follows: NFATc2 no. 1, 5′-AUGGAUUCUGGAGCCGAGUUUCUCC-3′; NFATc2 no.2, 5′-UUAAGGAUCCGCUCAUCAGCUGUCC-3′; NFATc1 no.1, 5′-AAACUGGUUAUUGUUGUGGUACAGG-3′; NFATc1 no.2, 5′-GCCAACGGUAACGCCAUCUUUCUAA-3′; NFATc1 no.3, 5′-CACUGAUGAUUAUGAGCCUGCUCCA-3′; and NFATc1 no.4, 5′-GCAUGAGGACGGUAGUCCUAAUUUG. As the control, Stealth RNAi negative control duplexes (Invitrogen Life Technologies) were used. An anti-Xpress Ab was purchased from Invitrogen Life Technologies, anti-NFATc2 and anti-NFATc1 Abs were from Santa Cruz Biotechnology, and an anti-CD3 Ab came from Janssen Pharmaceutica. The anti-NFATc3 (NFATx, NFAT4) Ab is described elsewhere (21). All other reagents were from Sigma-Aldrich.

Plasmid constructs

The PCR fragment of cDNA encoding full-length human $NFATc2_{aa\ 1-925}$, $NFATc1_{1-716}$, and their chimeric molecules NFATc1-c1-c2 ($NFATc1_{1-716}$-$NFATc2_{699-925}$), NFATc1-c2-c2 ($NFATc1_{1-419}$-$NFATc2_{395-925}$), NFATc2-c1-c2 ($NFATc2_{1-394}$-$NFATc1_{420-716}$-$NFATc2_{699-925}$), NFATc1-c1-c1/C ($NFATc1_{1-697}$-$NFATc1/C_{685-930}$), NFATc2-c2-c1/C ($NFATc2_{1-698}$-$NFATc1/C_{704-930}$), NFATc1-c2-c1/C ($NFATc1_{1-419}$-$NFATc2_{395-698}$-$NFATc1/C_{685-930}$), NFATc2/c2 ($NFATc2_{1-698}$), NFATc2/c1 ($NFATc2_{1-394}$-$NFATc1_{420-716}$), and NFATc1/c2 ($NFATc1_{1-419}$-$NFATc2_{395-698}$) or NFATc2-$TAD2_{699-925}$ was

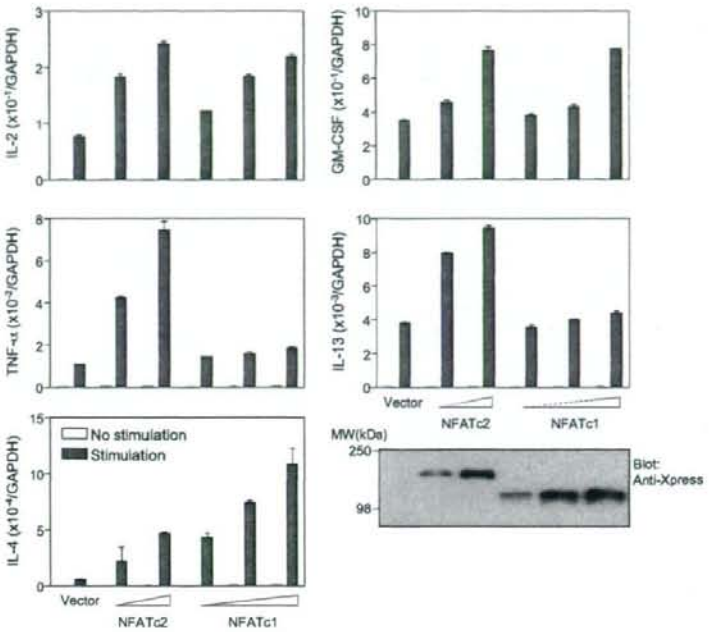

FIGURE 2. Effects of NFATc2 and NFATc1 on cytokine expression in T cells. Jurkat Tag cells were transfected with pEF-NFATc2 or pEF-NFATc1 or their empty vector (5–20 μg). At 48 h after transfection, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the expression of IL-2, TNF-α, IL-4, GM-CSF, and/or IL-13 mRNA was measured as shown in Fig. 1 (n = 4). The expression of NFATc2 and NFATc1 was analyzed by immunoblotting of whole cell lysates with an anti-Xpress Ab. The results shown are representative of at least three separate experiments.

subcloned in-frame into an appropriate site in the pEF6/His expression vector (Invitrogen Life Technologies). A mutant NFATc2 (NFATc2(KEF)) in which aa 914–916 (KEF) were exchanged for NDL and a mutant NFATc1-c1-c1/C (NFATc1-c1-c1/C(NDL)) in which aa 921–923 of NFATc1/C (NDL) were exchanged for KEF were also subcloned. In some experiments, the FLAG tag (MDYKDDDK) coding sequence was additionally subcloned in-frame into the resulting plasmid at the C-terminal end of the NFAT cDNA. The resulting NFATc2 and NFATc1 cDNA cassette, including the N-terminal Xpress-tag (DLYDDDDK), was cut out and subcloned into the CMV promoter-driven expression vector conjunct with an internal ribosomal entry site sequence (IRES) followed by the coding sequence of a luminescent protein, Venus, at the C-terminal end (22, 23). As reporter constructs, the 5′-flanking regions of human TNF-α (-670 to $+147$ relative to the transcription initiation site) and IL-2 (-418 to $+2$) genes were cloned into the pEGFP-1 vector (BD Bioscience Clontech). Six and five tandem repeats of the NFAT-binding site in the IL-2 promoter (-286 to -265) and the TNF-α promoter (-106 to -87), respectively, and three repeats of the AP-1 binding site in the metallothionein IIA gene (24, 25) and six repeats of the Jun/ATF2 binding site in the c-Jun promoter (AGCTAGCATTACCTCATCCCGATC) (26, 27) were subcloned into the pEGFP-1 vector in which the thymidine kinase minimum promoter sequence from the pRL-TK vector (Promega) was inserted. The correct sequences of all constructs were verified by sequencing.

Cells and transfection

With approval by the Ethical Review Committee of the Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan), $CD4^+$ T cells and $CD4^+CD45RO^-$ naive T cells were prepared from the peripheral blood of healthy volunteers by positive selection using a magnetic cell sorting system (Miltenyi Biotec). The purity of the resulting cells was >95% as determined by flow cytometry. Then, NFAT-IRES-Venus expression vectors were transfected into the cells by electroporation using a human T cell Nucleofector kit (Amaxa). The transfected cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1 × MEM nonessential amino acid solution, 100 U/ml penicillin G, and 100 U/ml streptomycin.

SV40 T Ag-transfected human leukemic Jurkat T (Jurkat Tag) cells were grown in the same medium as described above. Cells in the logarithmic growth phase were transfected with various amounts of plasmid DNAs by electroporation as described previously (25). In each experiment, cells in different groups were transfected with the same total amount of plasmid DNA by supplementing expression vector DNA with the proper amount of the corresponding empty vector. To introduce Stealth RNAi oligos into the cells, the same transfection was repeated after a 48-h interval. Immunoblotting against expressed and endogenous protein in whole cell lysates was performed as described previously (28).

Messenger RNA expression

At 48 h after final transfection, cells were treated with 2.5 μg/ml anti-CD3 Ab plus 1 μg/ml anti-CD28 Ab in the presence of 5 μg/ml anti-mouse IgG cross-linking Ab or 5 nM PMA plus ionomycin for 6 h at 37°C. In some experiments, stimulation was performed after the purification of transfection-positive cells, as determined by the fluorescence derived from Venus, by the FACSAria cell sorting system (BD Biosciences). The purity of sorted cells was >98% (data not shown). Quantitative real-time RT-PCR for TNF-α, IL-2, IL-4, IL-13, and GM-CSF was performed using Assay-on-Demand gene expression products (TaqMan MGB probes) with an ABI prism 7900 sequence detection system (Applied Biosystems) as described previously (28).

Intracellular cytokine staining

At 48 h after transfection, $CD4^+$ T cells were restimulated with 5 nM PMA plus 1 μM ionomycin for 6 h at 37°C in the presence of 2 μM monensin. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with PBS containing 3% BSA, cells were stained with anti-IL-2 (clone MQ1-17H12)-PE and anti-TNF-α (clone Mab11)-PE Abs (BD Biosciences). Flow cytometric analysis was performed on a FACSCalibur device with CellQuest software (BD Bioscience).

Reporter assay

After 16 h of stimulation, cytokine promoter-driven and transcription factor binding site-driven enhancer activity was assessed as the fluorescence of synthetic enhanced GFP (EGFP) detected by flow cytometry as described previously (28).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology) with slight modifications. Briefly, FLAG-tagged, NFAT expression vector-transfected Jurkat cells (2×10^7) were cultured with 5 nM PMA plus 1 μM

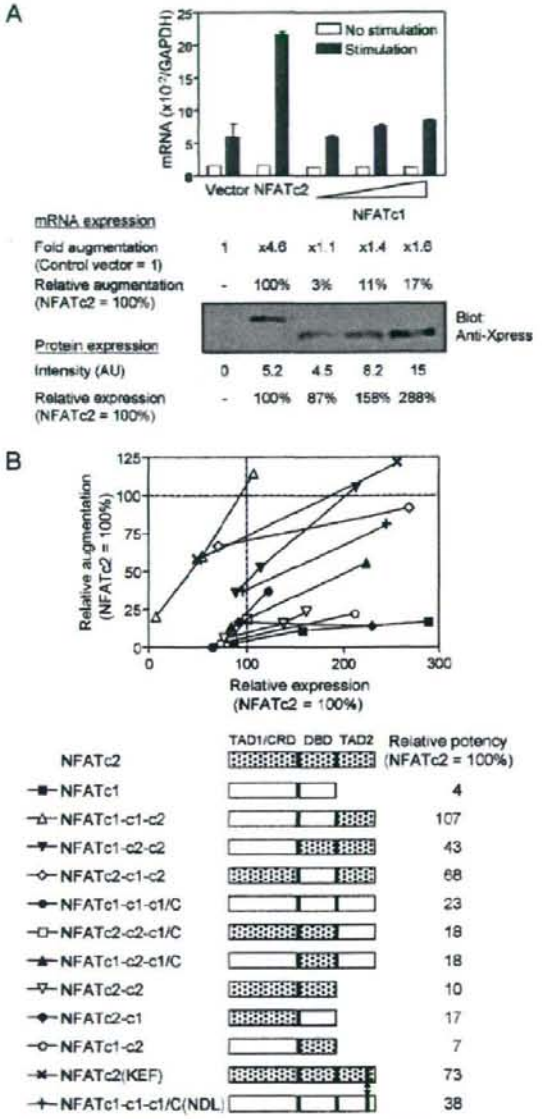

FIGURE 3. Relative effects of NFATc2/NFATc1 chimeric molecules on TNF-α gene expression. Jurkat Tag cells were transfected with pEF-NFATc2 or pEF-NFATc2/NFATc1 chimera or their empty vector (5–20 μg). At 48 h after transfection, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the expression of TNF-α mRNA was measured as shown in Fig. 1. The relative potency of NFATc1 to up-regulate TNF-α expression as well as the relative amount of expressed NFATc1 protein was determined in comparison with the effect of NFATc2 (*A*). The effects of chimeras on TNF-α expression were determined by their relative potency calculated from the protein expression-augmentation curves (*B*). The results shown are representative of at least two separate experiments. AU, Arbitrary units.

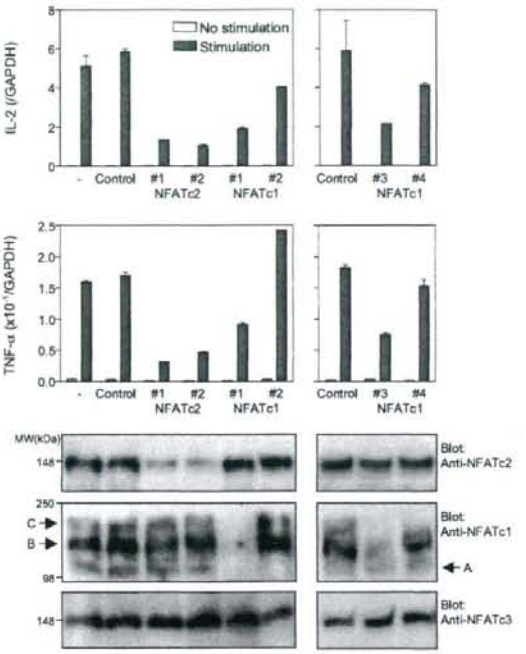

FIGURE 4. Knockdown effects of NFATc2 and NFATc1 on cytokine expression in T cells. Jurkat Tag cells were transfected with NFATc2- or NFATc1-specific Stealth RNAi oligos twice on day 0 and day 2. On day 4, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the expression of IL-2 and TNF-α mRNA was measured as shown in Fig. 1. The expression of NFATc2, NFATc1, and NFATc3 was analyzed by immunoblotting of whole cell lysates. Arrowheads indicate bands specific for isoforms A, B, and C of NFATc1. The results shown are representative of at least three separate experiments.

ionomycin for 30 min and then treated with 1% formaldehyde. After incubation for 30 min at room temperature, cells were washed twice, resuspended in 250 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A), and incubated on ice for 10 min. The lysates were sonicated three times for 20 s each, and then the debris was removed by centrifugation. One percent of the lysate was used as the DNA input control. The remaining samples were precleaned with salmon sperm DNA/protein A agarose slurry and then incubated with anti-FLAG (M2) agarose beads for 16 h at 4°C. The resulting beads were washed five times according to the manufacturer's protocol. The immunocomplexes were eluted and reverse cross-linked by incubation with 200 μl of elution buffer (1% SDS and 0.25 M NaCl) for 4 h at 65°C. The resulting DNA was subjected to analysis with a SYBR Green real-time PCR system (Takara) using primers specific for NFAT-IL-2 (5′ primer $^{-281}$AAAAACTGTTTCATACAGAAGGCGTTA^{-255} and 3′ primer $^{-141}$CTGATGACTCTTTGGAATTTCTTTA^{-165}) and NFAT-TNF-α (5′ primer $^{-228}$AGGATGGGGAGTGTGAGGG^{-210} and 3′ primer $^{-81}$CCTTGGTGGAGAAACCCATGAGCTCATCT^{-109}). The results were expressed as relative binding activity in comparison with the amount of input DNA. The detection limit of this assay, determined in the absence of anti-FLAG beads, was $<1 \times 10^{-5}$ of input DNA (data not shown).

Results

Differential contribution of NFATc2 and NFATc1 to cytokine expression in T cells

To compare the roles of NFATc2 and NFATc1 in cytokine synthesis by T cells, each NFAT expression vector constructed with IRES-Venus at the C-terminal end was transfected into human peripheral naive CD4$^+$ T cells. Twenty to 30% of the resulting cells were recognized to be transfection-positive, and the fluorescence levels were not significantly different among control vector-, NFATc2-IRES-Venus-, and NFATc1-IRES-Venus-transfected cells (Fig. 1*A*). Upon activation through the TCR and a costimulatory molecule, the mRNA of IL-2 and TNF-α was clearly increased in the

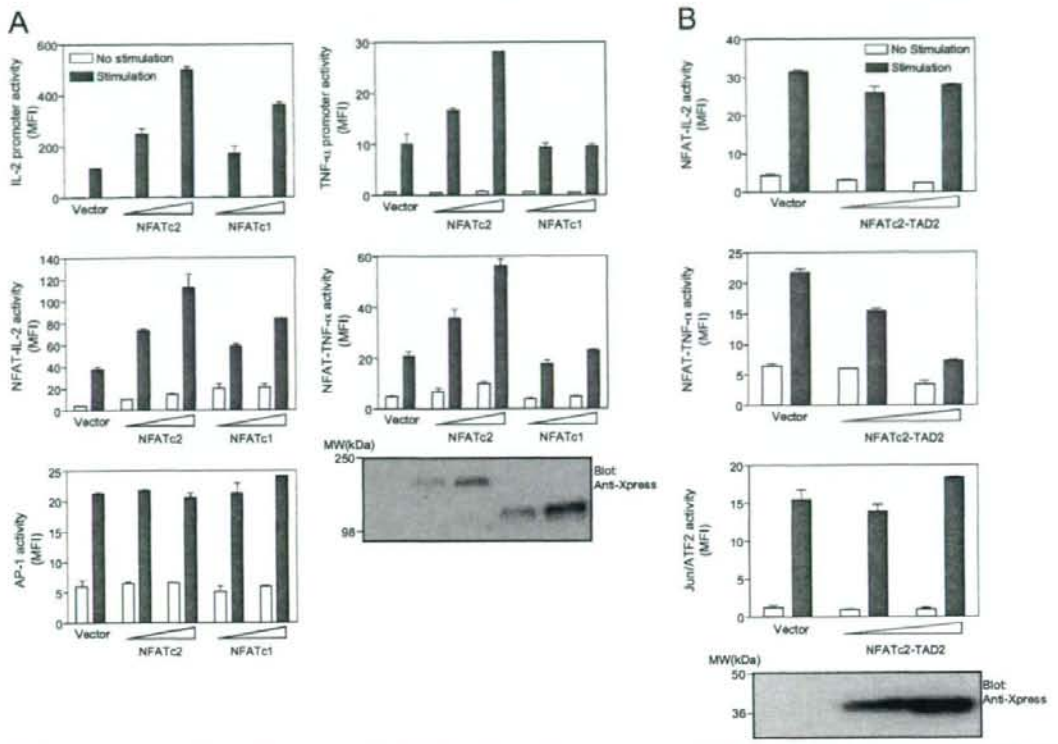

FIGURE 5. Effects of NFATc2 and NFATc1 on NFAT-dependent cytokine gene promoter activity in T cells. Jurkat Tag cells were transfected with the whole IL-2 and TNF-α promoter-, NFAT-IL-2-, NFAT-TNF-α-, AP-1- or Jun/ATF2-driven EGFP reporter vector in the presence of pEF-NFATc2, pEF-NFATc1 (*A*) or pEF-NFATc2-TAD (*B*), or their empty vector (5–10 μg). At 48 h after transfection, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 16 h, and the promoter activity was determined as the fluorescence of synthetic EGFP measured by flow cytometry. Data are shown as mean ± SEM of 2–4 experiments. The expression of introduced NFATc2, NFATc1, and NFATc2-TAD2 was analyzed by immunoblotting of whole cell lysates with an anti-Xpress Ab.

transfection-positive cells as determined by the fluorescence derived from Venus (Fig. 1*B*). Inducible IL-2 expression was up-regulated by NFATc2 and NFATc1. In contrast, NFATc2 but not NFATc1 significantly enhanced TNF-α expression in naive $CD4^+$ T cells (Fig. 1*B*). Western blot analysis confirmed that NFATc2 and the short form NFATc1 proteins were overexpressed in each transfection-positive cell population (Fig. 1*C*).

The production of IL-2 and TNF-α in the cells was further examined by intracellular staining. Forty to 50% of transfected cells produced IL-2 as well as TNF-α upon stimulation (Fig. 1*D*), whereas <1% were cytokine positive without stimulation (data not shown). In the population of transfection-positive cells, IL-2 and TNF-α production was up-regulated by NFATc2. NFATc1 also augmented IL-2 production by $CD4^+$ T cells, although the synthesis of TNF-α was not affected by the introduction of NFATc1 (Fig. 1*D*). These findings suggest that NFATc1 is defective in the transactivation of TNF-α in human $CD4^+$ T cells.

Next, the effects of NFATc2 and NFATc1 on the expression of several cytokines were comparatively analyzed. As shown in Fig. 2, Jurkat Tag cells expressed IL-4, IL-13, and GM-CSF along with IL-2 and TNF-α upon stimulation. Consistent with Fig. 1, increasing amounts of NFATc2 and NFATc1 overexpressed in the cells similarly up-regulated the expression of IL-2, as well as GM-CSF, in a dose-dependent manner. NFATc1 hardly affected the expression of not only TNF-α but also IL-13, although both were clearly enhanced by NFATc2. The up-regulation of IL-4 expression by NFATc1 was more potent than that by NFATc2 (Fig. 2). These findings suggest that the relative contribution of NFATc2 and NFATc1 differs among cytokines produced by T cells.

NFATc2-TAD2 is essential for transactivation of TNF-α

NFAT is composed of TAD1, followed by CRD, DBD, and TAD2. The shortest NFATc1 isoform is almost completely deficient in TAD2 (1, 17). To identify the responsible region causing the selective defect of NFATc1 in transactivation of the TNF-α gene, several NFAT chimeras in which functional domains were exchanged between NFATc2 and NFATc1 were used. For an accurate comparison of the potency of the expressed proteins, the density of the immunoblot bands was measured and the relative amount of expressed protein was determined in comparison with that of expressed NFATc2 as the control in a parallel experiment. At the same time, the relative potency of chimeric proteins as well as NFATc1 in the up-regulation of TNF-α expression was also assessed in comparison with that of NFATc2. Representative data in the case of NFATc1 are shown in Fig. 3*A*. Through the introduction of increasing amounts of chimeric protein-expression vectors, expression level augmentation curves were plotted for each chimera (Fig. 3*B*). Finally, the relative potency of chimeric proteins to transactivate the TNF-α gene at the same protein expression level as NFATc2 was calculated by extrapolation. The chimeras NFATc1-c1-c2, NFATc1-c2-c2, and NFATc2-c1-c2 substantially augmented TNF-α expression, whereas the effects of

NFATc2-c2, NFATc2-c1, and NFATc1-c2 as well as wild-type NFATc1 lacking TAD2 were very weak, suggesting that TAD2 is required for NFAT-mediated transactivation of TNF-α. It was also suggested that the functional difference between NFATc2 and NFATc1 is not essentially caused by the heterogeneity of their TAD1, CRD, and DBD domains. NFATc1 is composed of several isoforms mainly derived by alternative translation initiation and polyadenylation events (15–17). Interestingly, the potency of the longest form, NFATc1/C (NFATc1-c1-c1/C), and the chimeras NFATc2-c2-c1/C and NFATc1-c2-c1/C, which carry TAD2 of NFATc1/C to activate TNF-α, were weaker than that of the NFATc2-TAD2-combined proteins (Fig. 3*B*). These findings suggest that TAD2, especially that derived from NFATc2, is required for augmentation of TNF-α expression by NFAT.

It has been demonstrated that 15 amino acids in the C-terminal end of TAD2 are required for the maximum transactivation activity of NFAT (29). Therefore, we next examined the effects of mutants in which three amino acids in the corresponding region of NFATc2 (^{916}KEF^{918}) and NFATc1-c1-c1/C (^{921}NDL^{923}) were exchanged, on TNF-α expression. As shown in Fig. 3*B*, the replacement of KEF by NDL in NFATc2 and that of NDL by KEF in NFATc1-c1-c1/C little repressed and augmented, respectively, their transactivation activity for TNF-α.

Knockdown effects of NFATs on cytokine expression

The roles of endogenously expressed NFATc2 and NFATc1 in cytokine expression were next examined by using RNAi technology. The expression of IL-2 and TNF-α in Jurkat Tag cells was similarly diminished by the introduction of two independent Stealth RNAi oligos against NFATc2, along with selective down-regulation of NFATc2 protein expression in the cells (Fig. 4). The expression of all NFATc1 isoforms in Jurkat Tag cells was down-regulated by the introduction of one NFATc1-Stealth RNAi oligo (no.1). In contrast another oligo (no.2), derived from a sequence close to the proximal poly(A) signal and not completely overlapping the sequences of longer isoforms, specifically diminished the expression of the shortest form of NFATc1 (Fig. 4, isoform A). The knockdown of all NFATc1 isoforms inhibited the expression of IL-2 as well as that of TNF-α. However, down-regulation of the shortest isoform alone resulted in a slight decrease in IL-2 but weak augmentation rather than suppression of TNF-α. In addition, successful knockdown of NFATc1/B plus NFATc1/C and NFATc1/C alone by the no. 3 and no.4 oligos induced a 50 ~ 60% and 10 ~ 20% reduction, respectively, of IL-2 and TNF-α expression. These findings are consistent with the results obtained by overexpression studies (Figs. 1–3) and suggest that the shortest NFATc1, like other isoforms and NFATc2, plays a positive role in IL-2 expression, although this isoform does not contribute as a transcription activator of TNF-α in physiological conditions.

Differential effects of NFATc2 and NFATc1 on cytokine promoters

To investigate the mechanisms by which NFATc2 and NFATc1 differentially affect the expression of IL-2 and TNF-α, a promoter reporter assay was performed. In agreement with the results of mRNA expression as shown in Fig. 2, inducible IL-2 promoter activity was enhanced by NFATc2 and NFATc1, whereas NFATc2 but not NFATc1 augmented TNF-α promoter activity (Fig. 5*A*). Furthermore, a difference between NFATc2 and NFATc1 was also observed in the reporter assay using the NFAT binding sites in the IL-2 and TNF-α gene promoters (NFAT-IL-2 and NFAT-TNF-α, respectively). Thus, the enhancer activity of NFAT-IL-2 was up-regulated by NFATc2 and NFATc1, whereas that of NFAT-TNF-α was augmented by NFATc2 but not by

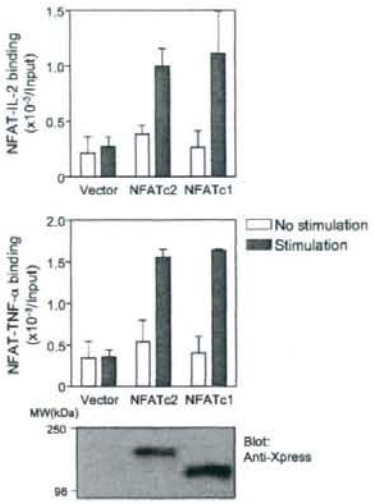

FIGURE 6. Association of NFATc2 and NFATc1 with cytokine promoters. Jurkat Tag cells were transfected with pEF-NFATc2-FLAG, pEF-NFATc1-FLAG, or their empty vector (10 μg). At 48 h after transfection, formaldehyde cross-linked chromatin prepared from cells upon stimulation with or without 5 nM PMA plus 1 μM ionomycin for 30 min was immunoprecipitated with an anti-FLAG Ab. Input and precipitated DNA were analyzed by SYBR Green real-time PCR. Binding activity is expressed as the relative amount of precipitated DNA in comparison with the input control DNA. Expression of introduced NFATc2 and NFATc1 was analyzed by immunoblotting of whole cell lysates with an anti-Xpress Ab. The results shown are mean ± SEM of three separate experiments.

NFATc1 (Fig. 5*A*). Neither NFATc2 nor NFATc1 affected AP-1-derived activity, suggesting that the distinct effects of NFATc2 and NFATc1 on IL-2 and TNF-α expression were due, at least in part, to the effects on the corresponding binding region in their respective promoters.

To further investigate the role of TAD2 in TNF-α gene transcription, the effect of the TAD2 domain on NFAT-dependent transcriptional activity was examined. As shown in Fig. 5*B*, ectopically expressed NFATc2-TAD2 behaved as a dominant negative in the transcriptional activity driven by the NFAT binding site in the TNF-α promoter. Thus, transfected NFATc2-TAD2 suppressed inducible NFAT-TNF-α activity in a dose-dependent manner, whereas NFAT-IL-2 activity was not affected. These results support the notion that TAD2 is important for transactivation of the TNF-α gene but not the IL-2 gene by NFAT.

It has been demonstrated that NFAT cooperates with the Jun/ATF2 heterodimer for the transactivation of TNF-α (30–32). Therefore, we next examined the effect of NFATc2-TAD2 on Jun/ATF2-dependent transcriptional activity. As shown in Fig. 5*B*, inducible Jun/ATF2 activity was not affected by ectopically expressed NFATc2-TAD2, suggesting at least that the NFATc2-TAD2 domain, by itself, does not obstruct the association of Jun/ATF2 with their recognition sequence and/or the transactivation of Jun/ATF2-responsive genes.

NFATc2 and NFATc1 equivalently associate with NFAT-TNF-α

The findings of the reporter assay raise the possibility that interactions with NFAT sites in the IL-2 and TNF-α promoters differ between NFATc2 and NFATc1. A difference in the binding activity for cytokine promoters among NFAT family members has been suggested (18) although not fully elucidated, especially in physiological conditions. The next examination was, therefore, performed to compare the