高橋秀実

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
高橋秀実	特異免疫およびその賦活法に関する基本原理	林英生、岩本愛吉、神谷茂、高橋秀実	ブラック微生物学	丸善出版	東京	2007	495-533

雑誌

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高橋秀実	第５回日本中医学交流会大会：感染症に対する温病治療-SARS は攻略できるか.	中医臨床	28	374-379	2007
高橋秀実	ワクチンによる特異的免疫機能の誘導：ヒトにおける抗原特異的免疫機構.	治療学	41	1041-1045	2007
高橋秀実	γδT細胞とリウマチ様関節炎.	リウマチ科	38	565-570	2007
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高橋秀実	免疫応答とエネルギーのめぐり.	癒しの環境	13	印刷中	2008
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高橋めぐみ、高橋秀実	遊離抗原によるCD8+T細胞のアポトーシス誘導.	臨床免疫・アレルギー科		印刷中	2008
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横田恭子

研究成果の刊行に関する一覧表

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Takayanagi, R., Ohashi, T., Yamashita, E., Kurosaki, Y., Tanaka, K., Hakata, Y., Komoda, Y., Ikeda, S., Tsunetsugu-Yokota, Y., Tanaka, Y., H. Shida.	Enhanced Replication of Human T-cell Leukemia Virus Type 1 in T Cells from Transgenic Rats Expressing Human CRM1 That Is Regulated in a Natural Manner	J. Virol.	81	5908-5918	2007
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IV. 研究成果の刊行物・別刷

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.

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[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.

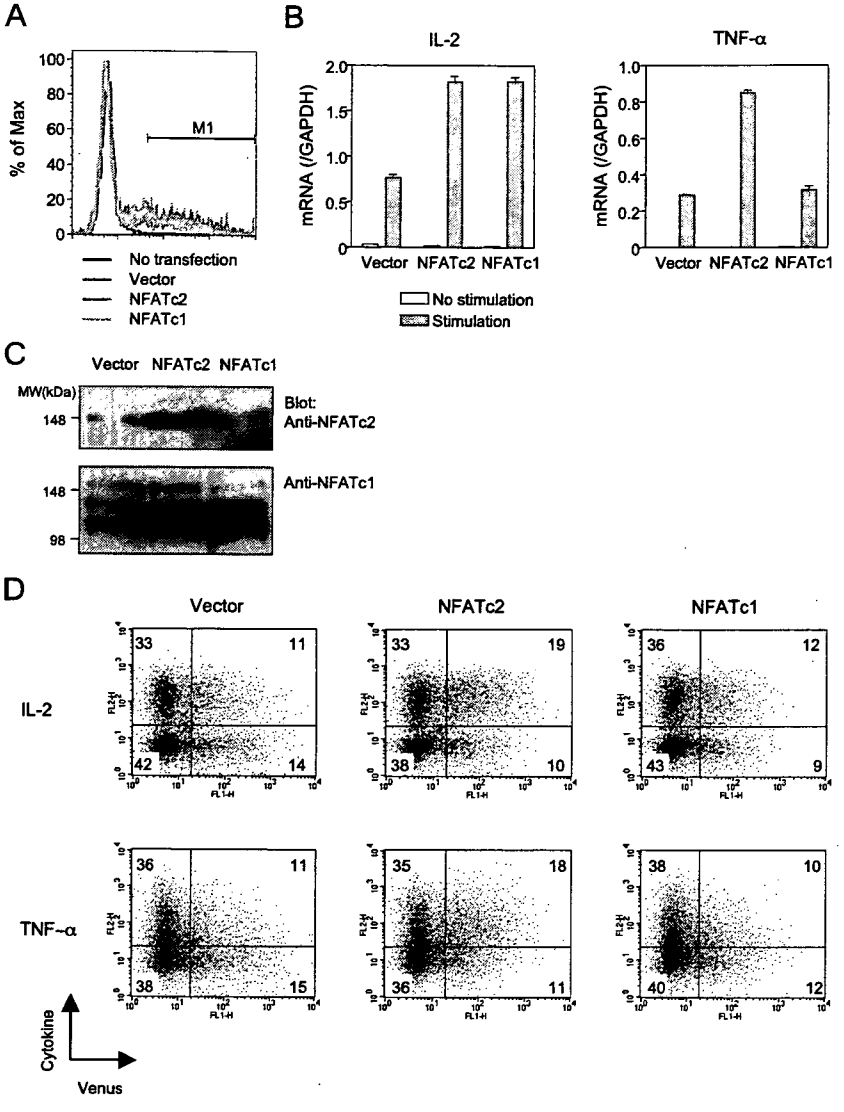

FIGURE 1. Effects of NFATc2 and NFATc1 on cytokine synthesis by T cells. Human naive or total $CD4^+$ T cells were transfected with NFATc2- or NFATc1-IRES-Venus or their empty vector (5 μg). At 48 h after transfection, transfection-positive naive $CD4^+$ T cells (M1 population) (*A*) were purified and 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′-GCCAA CGGUAACGCCAUCUUUCUAA-3′; NFATc1 no.3, 5′-CACUGAUGAUU AUGAGCCUGCUCCA-3′; and NFATc1 no.4, 5′-GCAUGAGGACGGUAG UCCUAAUUUG. 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

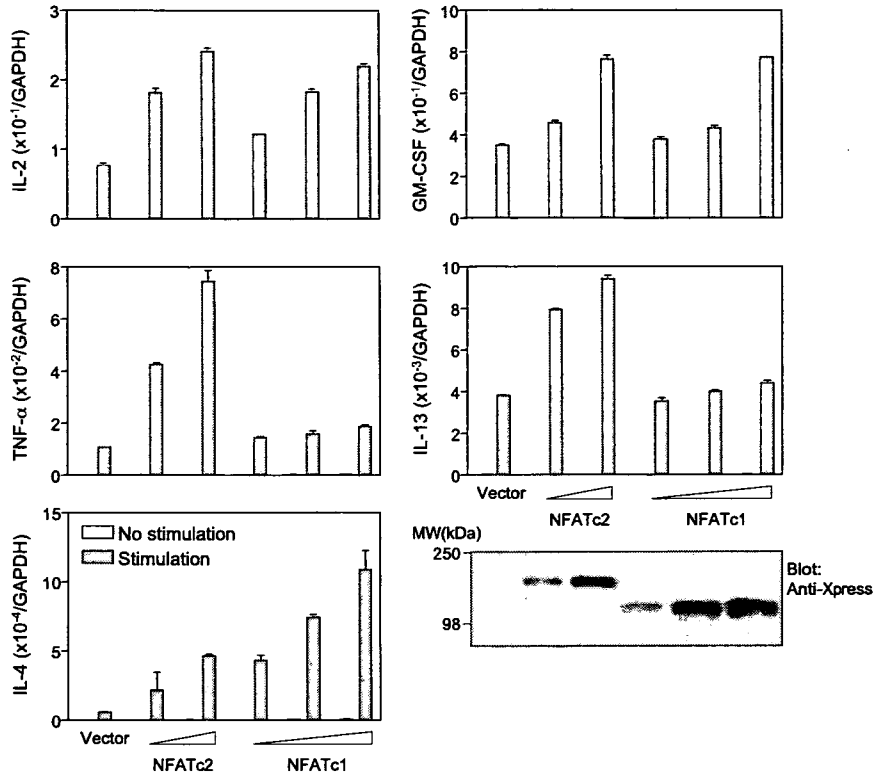

FIGURE 2. Effects of NFATc2 and NFATc1 on cytokine expression in T cells. Jurkat Tag cells were transfected with pEF-NFATc2 or pEF-NFATc1 or their empty vector (5–20 μg). At 48 h after transfection, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the expression of IL-2, TNF-α, IL-4, 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

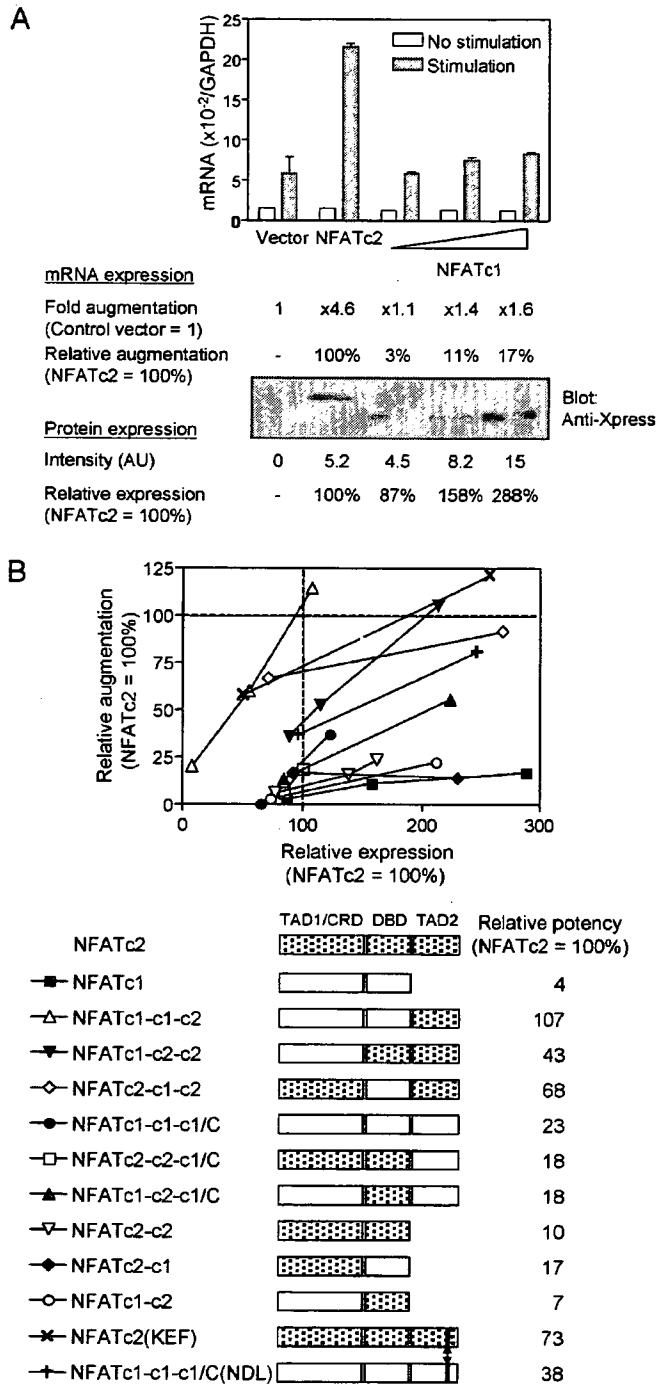

FIGURE 3. Relative effects of NFATc2/NFATc1 chimeric molecules on TNF-α gene expression. Jurkat Tag cells were transfected with pEF-NFATc2 or pEF-NFATc2/NFATc1 chimera or their empty vector (5–20 μg). At 48 h after transfection, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the 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.

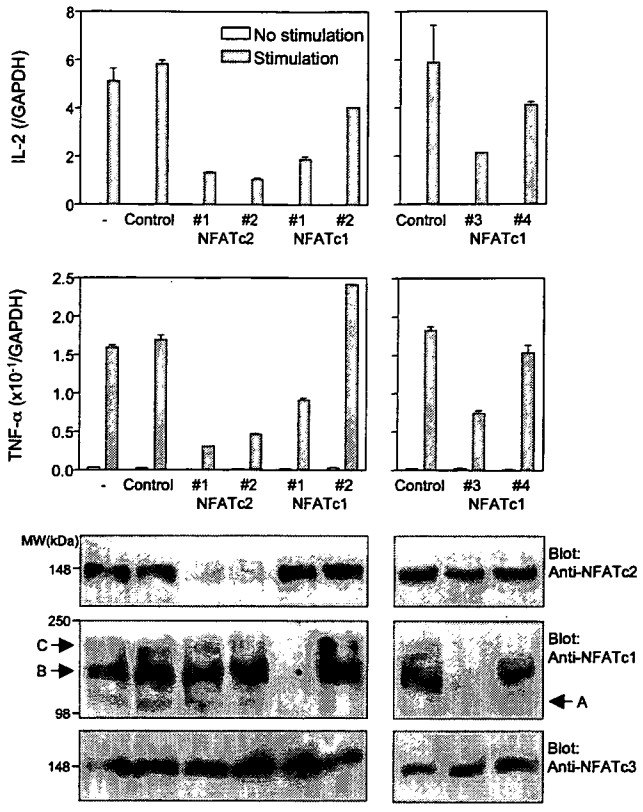

FIGURE 4. Knockdown effects of NFATc2 and NFATc1 on cytokine expression in T cells. Jurkat Tag cells were transfected with NFATc2- or NFATc1-specific Stealth RNAi oligos twice on day 0 and day 2. On day 4, cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the expression of IL-2 and 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

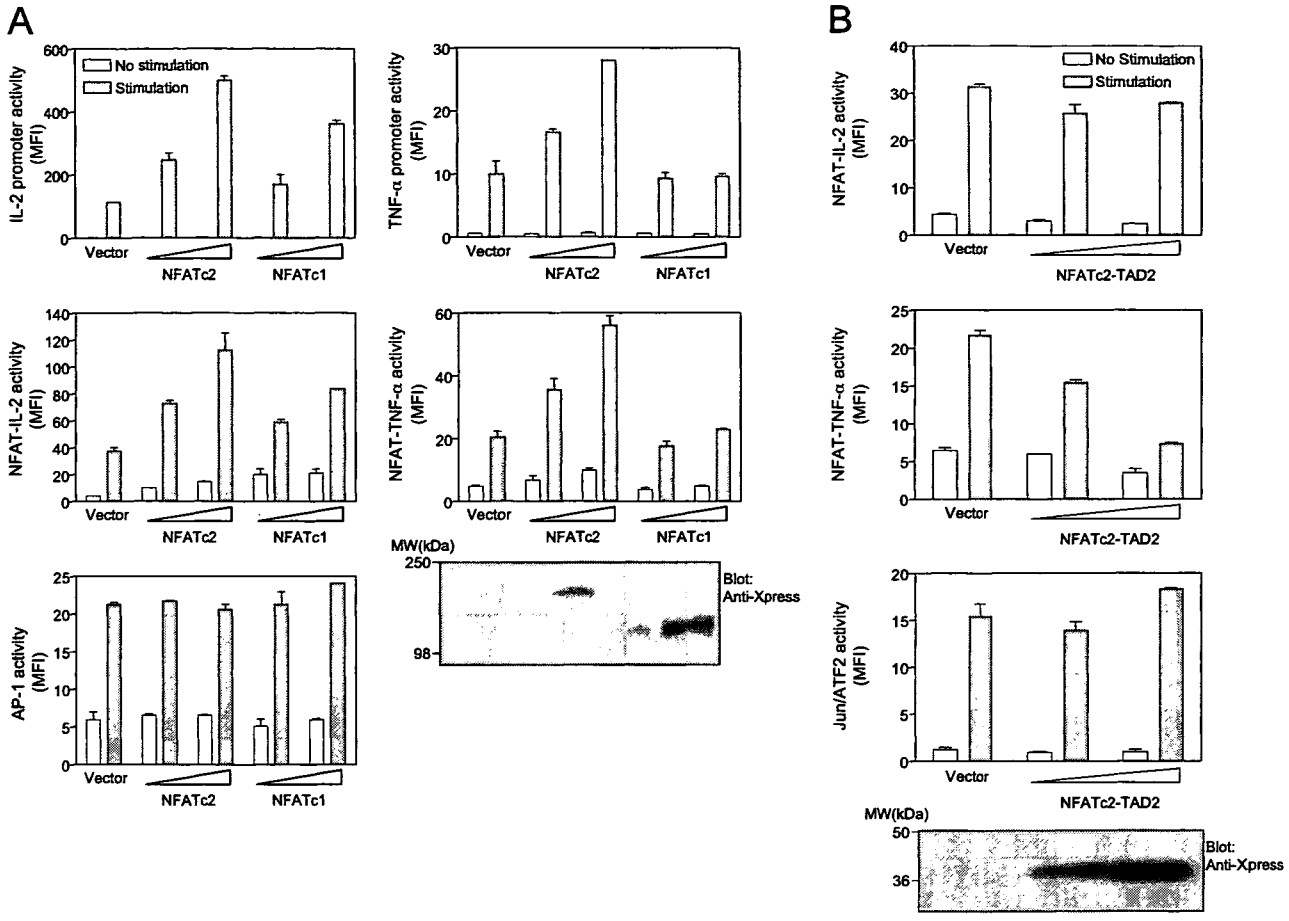

FIGURE 5. Effects of NFATc2 and NFATc1 on NFAT-dependent cytokine gene promoter activity in T cells. Jurkat Tag cells were transfected with the whole IL-2 and TNF-α promoter-, NFAT-IL-2-, NFAT-TNF-α-, AP-1- or Jun/ATF2-driven EGFP reporter vector in the presence of pEF-NFATc2, pEF-NFATc1 (*A*) or pEF-NFATc2-TAD (*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

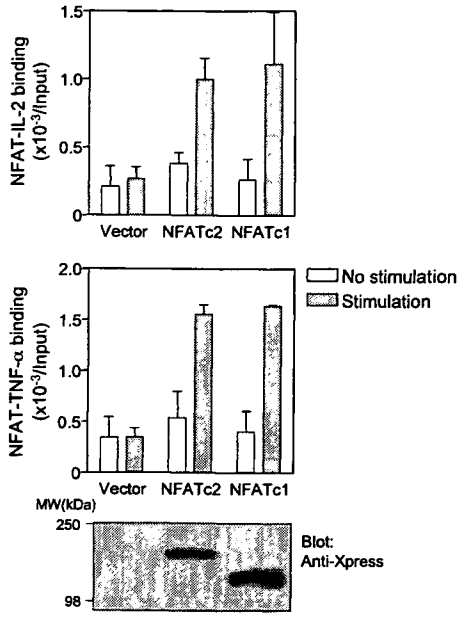

FIGURE 6. Association of NFATc2 and NFATc1 with cytokine promoters. Jurkat Tag cells were transfected with pEF-NFATc2-FLAG, pEF-NFATc1-FLAG, or their empty vector (10 μg). At 48 h after transfection, formaldehyde cross-linked chromatin prepared from cells upon stimulation with or without 5 nM PMA plus 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

binding properties of NFATc2 and NFATc1 to the IL-2 and TNF-α promoters by using a ChIP assay. As shown in Fig. 6, almost the same amounts of the IL-2 and TNF-α promoter regions were coprecipitated with ectopically expressed NFATc2 as well as NFATc1 in Jurkat Tag cells upon stimulation. These results suggest that the selective defect of NFATc1 in up-regulation of TNF-α was not because of lack of binding activity for the TNF-α promoter/enhancer region.

Discussion

The differential contribution of NFATc2 and NFATc1 to TNF-α gene transcription has been suggested by a reporter assay and an in vitro DNA binding assay (15, 18, 19). This study revealed that NFATc2, but not NFATc1, promoted TNF-α synthesis in human peripheral $CD4^+$ T cells. This is generally consistent with previous reports that TNF-α synthesis by lymphocytes and/or T cells was impaired in NFATc2$^{-/-}$ mice (9) but not in NFATc1$^{-/-}$ mice (8). Furthermore, our present results demonstrating that the enhancement of TNF-α expression by NFAT was lost by the truncation of TAD2 and that NFATc2-TAD2 specifically suppressed NFAT-TNF-α activity clearly suggest that TAD2 is required for NFAT-mediated transactivation of the TNF-α gene but not the IL-2 gene.

It has been demonstrated that the full response at many NFAT sites requires concomitant activation of the AP-1 transcription factor family (1, 33). There is a discernible AP-1 site immediately downstream of the NFAT sites in the promoter region of IL-2 and other cytokine genes. Furthermore, the three-dimensional structures of NFATc2-DBD, AP-1 heterodimer (Jun/Fos), and the distal Ag-receptor response element in the IL-2 gene promoter, which we used as NFAT-IL-2 in this study, have been analyzed in detail (34). The importance of NFAT/AP-1 cooperativity in gene regulation was revealed by studies demonstrating that mutations of the AP-1-interacting domain of NFATc2 led to loss of transactivation of many cytokine genes, including IL-2 (35). Deletion of either the NFAT site or the AP-1 site is sufficient to destroy IL-2 promoter activity (36).

In contrast, the association of NFAT with the consensus sequence for a NFκB-binding site spanning −106 to −87 in the TNF-α gene promoter (κ3 site, which we used as NFAT-TNF-α in this study) is essential for TNF-α expression in T cells (37–39). Unlike the case of IL-2, Macian et al. (35) demonstrated that cooperation with AP-1 is not crucial for NFAT-dependent transactivation of the TNF-α gene. Cooperation with the Jun/ATF2 heterodimer, but not with AP-1 (Jun/Fos), is required for full induction of TNF-α expression by NFAT (30–32), although the Jun/ATF2-interacting region in NFAT has not been identified. In contrast to NFAT-IL-2 on which NFAT contacts AP-1 via its DBD, the following evidence suggests the possibility that NFAT interacts with Jun/ATF2 or other undefined coactivators on NFAT-TNF-α through TAD2. First, TAD2 was crucial for NFAT-dependent transactivation of the TNF-α gene (Fig. 3). Second, TAD2 behaved as a dominant negative against the transcriptional activity of NFAT-TNF-α, but not against that of NFAT-IL-2 (Fig. 5*B*). In addition, NFATc2 and NFATc1 equivalently associated with NFAT-TNF-α in vivo (Fig. 6). Nevertheless, Jun/ATF2-dependent transcriptional activity was not affected by NFATc2-TAD2 (Fig. 5*B*). Therefore, NFAT may cooperate with other factors than Jun/ATF2 in activating NFAT-TNF-α; otherwise, similar to the case of NFAT/AP-1 complex (34), strong interaction between NFAT and Jun/ATF2 may require their target DNA sequences. To elucidate additional details of the contribution of TAD2 to NFAT-TNF-α activation, analysis of the cocrystallized structure of the complex of transcription factors, including NFAT, on NFAT-TNF-α may be required as performed for the NFAT/AP-1/NFAT-IL-2 complex (34).

However, in disagreement with our present findings (Fig. 6), it has been reported that binding activity for NFAT-TNF-α was detectable in nuclear extracts of NFATc2- but not NFATc1- or NFATc3-transfected COS cells (18). In addition, Oum et al. (19), using in vitro DNA-protein binding assay and a reporter assay with HeLa cells, demonstrated that the functional disparity between NFATc2 and NFATc1 in the activation of TNF-α was due to the different binding specificity of NFATc2 and NFATc1 to NFAT-TNF-α. The reason for the discrepancy is unclear; our present data using NFATc2/NFATc1 chimeric molecules (Fig. 3) further support the notion that TAD2 rather than DBD predominantly contributes to the difference in TNF-α transactivation activity between NFATc2 and NFATc1. The condition in which the transcription complex was formed in vivo might not have been completely reproducible in the in vitro binding assay. Furthermore, interaction of transcription factors with their corresponding sequences may have differed among the cell types used. In fact, Monticelli et al. (40) reported that the association of NFATc1 with the murine IL-13 promoter was different between Th2 cells and mast cells. Therefore, our results showing that NFATc2 and NFATc1 equivalently associated with NFAT-TNF-α, demonstrated in T cells by ChIP assay, seem to be relatively reliable even though the physiological association between endogenous NFAT and the TNF-α promoter was too weak to further confirm this in our experimental conditions (data not shown).

In agreement with the notion that TAD2 is required for NFAT-TNF-α activation, positive participation of longer forms of NFATc1, which have TAD2, in TNF-α expression was suggested by the fact that reductions of all three NFATc1 isoforms by RNAi resulted in slight suppression of TNF-α, whereas the introduction of Stealth RNAi oligo, which down-regulated the shortest form of NFATc1 alone, failed to do so (Fig. 4). We also observed that TNF-α expression was enhanced by the longest NFATc1 isoform, although its potency was relatively weaker than that of NFATc2 (Fig. 3). These findings are partly consistent with a previous report demonstrating that the potency of NFATc.β, which has medium-length TAD2, to enhance NFAT-TNF-α activity was very low (15) and suggesting that the contribution of TAD2 derived from NFATc1 to TNF-α expression is smaller than that of NFATc2-TAD2. The essential domain in NFAT-TAD2 for its transactivation activity has not been fully elucidated. Imamura et al. (29) demonstrated that 15 amino acids, DITLDDVNEIIGRDM, in the C-terminal end of NFATx1 are required for its maximum transactivation activity in Jurkat T cells. In that region, the first 12 amino acids, DITLDDVNEIIG, are conserved in NFATc2$_{904-915}$ as well as NFAT1c/C$_{909-920}$. Exchange of the remaining three amino acids in NFATc2 (KEF) and NFATc1-c1-c1/C (NDL) little suppressed and enhanced, respectively, their transactivation activity, suggesting that the corresponding region is responsible for the differential participation of the TAD2 of NFATc2 and NFATc1/C origins. Because the effects of exchange were weak, the existence of another unknown region in TAD2 contributing to the transactivation activity is also suggested. Further examination will be required to elucidate the differential roles of TAD2 derived from NFATc2 and NFATc1/C in detail. Above all, NFATc1, especially its major and inducible shorter form, potentially contributes to the clonal expansion of activated T cells through IL-2 synthesis, but not to TNF-α-mediated exacerbation of inflammation.

Differential effects of NFATc2 and NFATc1 on the expression of IL-13 were also observed, although GM-CSF expression was enhanced by both NFATs (Fig. 2). These findings are consistent with previous reports demonstrating that the effect of NFATc1 on IL-13 gene transcription was weaker than that of NFATc2, even though this was shown in mast cells rather than Jurkat cells (40). Accordingly, IL-13 expression in mast cells was unchanged in

NFATc1$^{-/-}$ mice (40). In the report of Macian et al. (35), augmentation of the promoter activity of IL-2 and GM-CSF by mutant NFATc2, which was unable to interact with AP-1, was weaker than that by wild-type NFATc2 even though the effects of both NFATc2s on TNF-α and IL-13 were equivalent. Similar to the case of IL-2, GM-CSF promoter activity was diminished by truncation of the AP-1 binding region neighboring the NFAT site (41). Taking these findings together with our present results, it is likely that NFATc1 activates GM-CSF in cooperation with AP-1, although TAD2 is required for transactivation of the IL-13 gene by NFAT through association with other cofactors. Because no sequence completely matching the NFAT-binding region in the TNF-α promoter was detectable in the 5′-flanking region of the IL-13 gene, the mechanisms by which NFATc1 fails to enhance IL-13 expression need to be further investigated.

In conclusion, TAD2 is required for NFAT-mediated transactivation of the TNF-α gene in T cells. NFATc1, especially its shortest isoform that lacks TAD2 and is predominantly induced upon cell activation, may play characteristic roles in immune responses through its specific activation of distinct cytokines. Our present findings will be useful for developing selective TNF-α inhibitors that will be promising drugs for treating autoimmune diseases, including rheumatoid arthritis.

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Disclosures

The authors have no financial conflict of interest.

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Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination

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Capable of inducing antigen-specific immune responses in both systemic and mucosal compartments without the use of syringe and needle, mucosal vaccination is considered ideal for the global control of infectious diseases. In this study, we developed a rice-based oral vaccine expressing cholera toxin B subunit (CTB) under the control of the endosperm-specific expression promoter 2.3-kb glutelin *GluB-1* with codon usage optimization for expression in rice seed. An average of 30 μg of CTB per seed was stored in the protein bodies, which are storage organelles in rice. When mucosally fed, rice seeds expressing CTB were taken up by the M cells covering the Peyer's patches and induced CTB-specific serum IgG and mucosal IgA antibodies with neutralizing activity. When expressed in rice, CTB was protected from pepsin digestion *in vitro*. Rice-expressed CTB also remained stable and thus maintained immunogenicity at room temperature for >1.5 years, meaning that antigen-specific mucosal immune responses were induced at much lower doses than were necessary with purified recombinant CTB. Because they require neither refrigeration (cold-chain management) nor a needle, these rice-based mucosal vaccines offer a highly practical and cost-effective strategy for orally vaccinating large populations against mucosal infections, including those that may result from an act of bioterrorism.

mucosal immunity | protein body | oral vaccine | IgA | cholera toxin B subunit

The majority of emerging and reemerging infectious pathogens, including *Vibrio cholerae*, *Escherichia coli*, HIV, influenza virus, or coronavirus causing severe acute respiratory syndrome, invade and infect the host via the mucosal surfaces of the gastrointestinal, respiratory, and/or genitourinary tracts (1–3). Mucosal immunity forms a first line of defense by means of secretory IgA and cytotoxic T cells against epithelium-transmitted pathogens, and so it would seem important to develop vaccines that induce effective immune responses at mucosal barriers. Most current vaccines are administered by needle and syringe, generating effective antibody and cell-mediated responses in the systemic compartment, but not in mucosal sites (4). In contrast, mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses in both systemic and mucosal compartments (5–8). Because it elicits this two-layered protective immunity, mucosal vaccination is thought to be an ideal strategy for combating both emerging and reemerging infectious diseases (5–8). In fact, the Bill and Melinda Gates Foundation and the National Institutes of Health have proposed that mucosal vaccines be a focus of future vaccine development (9), a vision underlying the foundation of the Gates' research initiative, "Grand Challenges in Global Health" (9). Most traditional vaccines are not cost-effective because they cannot be stored at room temperature (RT), instead requiring that the "cold chain" be preserved en route from vaccine manufacturer to the field of vaccination (i.e., that no gap be allowed in the refrigeration) (10). The cost of preserving that cold chain for currently used vaccines is estimated at between $200 and $300 million a year (10). Further, if inappropriately processed or disposed of, the needles and syringes used for the vaccination can pose the threats of environmental contamination and second-hand spread of infectious disease. Producing vaccine antigens in plants would offer many practical advantages (11, 12). First, it would be less expensive to produce vaccine antigens in plants than via industrial fermentation. Second, there is no need to take elaborate means to purify the vaccine if it is expressed in plant tissue. Third, the plant expression system minimizes risks arising from contamination. Collectively, these advantages make a plant-based subunit vaccine not only attractive, but also practical for the propagation of mucosal vaccine on the global scale (12).

As early as 1990, Curtiss and Cardineau expressed *Streptococcus mutans* surface protein antigen, the causative epitope for dental caries, in tobacco as a first step toward a potential plant-based mucosal vaccine (13). Since then, many vaccine antigen candidates, including bacterial diarrhea antigens, hepatitis B antigen, Norwalk virus antigen, and respiratory syncitial virus antigen, have been expressed in tobaccos or potatoes to demonstrate the feasibility of edible plant-based vaccines (14–21). However, these plant-based vaccines have remained a function of sophisticated bench-driven experiments and have not yet advanced to practical application. If such a vaccine is to be practicable for global immunization, it must be storable at RT for long periods, be protected from the harsh environment of the gastrointestinal tract, and target mucosal inductive tissues, including Peyer's patches (PPs) (8, 22).

We here introduce a rice-based oral vaccine possessing many practical advantages over most traditional or other plant-based oral vaccines. The rice-based oral vaccine is stable at RT for several years and is protected from digestive enzymes. When ingested, this vaccine induced antigen-specific antibodies with neutralizing activities. These results show that the rice-based oral vaccine offers a highly practical global strategy for cold-chain- and needle-free vaccination against infection.

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Abbreviations: CHO, Chinese hamster ovary; CT, cholera toxin; CTB, cholera toxin B subunit; LTB, heat-labile enterotoxin B subunit; PPs, Peyer's patches; PB, protein body; RT, room temperature.

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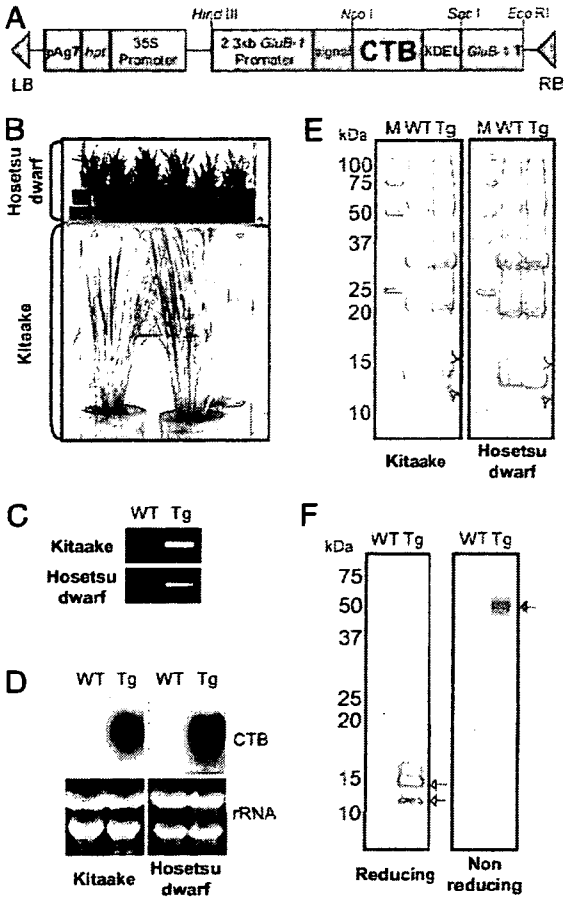

Fig. 1. Expression of CTB in transgenic rice. (*A*) T-DNA plasmid-inserted, codon-optimized CTB gene for rice seed, controlled by the rice seed storage protein glutelin 2.3-kb *GluB-1* promoter. The signal sequence of *GluB-1* and the retention signal to the endoplasmic reticulum coding KDEL are located at the N- and C-terminal regions, respectively. (*B*) The Kitaake and Hosetsu dwarf type rice strains expressed CTB in a closed chamber. (*C*) Integration of the CTB gene into the genomic DNA was confirmed by PCR. WT, Wild-type nontransgenic rice; Tg, CTB-expressed transgenic rice. (*D*) Northern blot analysis was performed for the confirmation of CTB mRNA expression. (*E* and *F*) SDS/PAGE and Western blot analysis revealed that high levels of CTB protein were expressed in rice. Arrowheads indicate 12- and 15-kDa forms of CTB (*E*). The CTB protein, composed of two fragments, forms a 55- to 65-kDa pentamer structure under nonreducing conditions. Arrows indicate monomeric (under reducing condition) and pentamer (under nonreducing condition) forms of CTB (*F*).

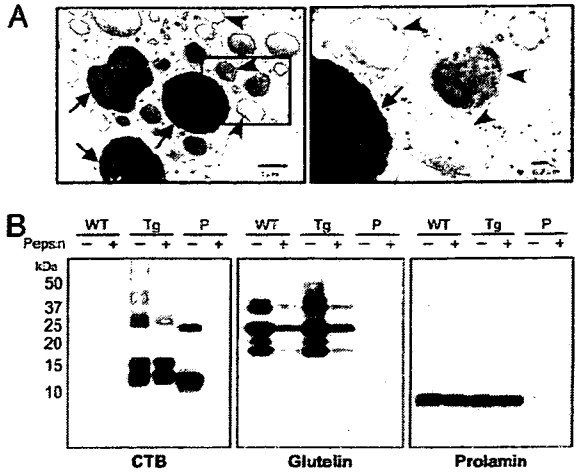

Fig. 2. Localization and digestive enzyme resistivity of rice-expressed CTB in PBs. (*A*) Data obtained through immunoelectron microscopic analysis with anti-CTB antibody. A positive signal was obtained with 20 nm of gold particles. CTB expressed in rice are stored in PB-I (arrowheads) and PB-II (arrows). (*B*) Pepsin digestion was carried out under the conditions described in *Materials and Methods*. Approximately 90% of glutelins but not 13k prolamins were digested *in vitro*, whereas ≈75% of rice-based CTB but not purified rCTB remained intact.

Results

Development of Rice-Based Mucosal Vaccine Expressing Cholera Toxin B Subunit (CTB) in Seeds. We purposely chose CTB as a prototype antigen to demonstrate both the capacity of the rice-based mucosal vaccine to induce systemic as well as mucosal immunity and to showcase the practicality of using the rice transgenic expression system. Once generated with binary vector (pGPTV-35S-HPT) (23), as described in Fig. 1*A*, codon-optimized CTB genes for rice seed were transfected into the rice plant [*Oryza sativa* L. cv Kitaake, a normal-sized rice (24); and Hosetsu, a dwarf type rice (25, 26), shown in Fig. 1*B*] by using *Agrobacterium tumefaciens*-mediated transformation (27). Hosetsu dwarf type rice, a naturally occurring gene mutant on the gibberellin biosynthesis pathway (25), is 20 cm in height and has a short life cycle (≈3 months). Genomic PCR analysis revealed that the CTB gene was integrated into the genomic DNA of both lines of rice plants (Fig. 1*C*). In addition, high levels of CTB-specific mRNA in the seeds of both lines of transgenic but not nontransgenic wild-type (WT) rice were expressed at 15 days after flowering (Fig. 1*D*). When we examined the accumulation of CTB protein in the transgenic rice seed by SDS/PAGE and Western blot analysis with anti-CTB polyclonal antibody, two bands (12 kDa and 15 kDa) were detected under denatured conditions (Fig. 1 *E* and *F*). Using densitometry analysis with rCTB as a standard, we found that expression levels of CTB reached an average of 30 μg per seed in the Kitaake strain, representing 2.1% of the total seed protein (0.15% of seed weight). The expression level of CTB in Hosetsu dwarf type rice was lower (average of 5 μg per seed) than that in Kitaake rice. Furthermore, Western blot analysis under nonreducing conditions revealed that CTB expressed in rice formed a pentamer with 55 to 65 kDa (Fig. 1*F*), indicating that most of the CTB expressed in rice seed is considered to be a functionally native form possessing the ability to bind to the GM1 ganglioside, known to be expressed at the apical surface of the intestinal epithelium and to be a receptor for CTB (28).

CTB Expressed in Protein Bodies (PBs) of Rice Seed Is Resistant to Gastrointestinal Harsh Environment. In addition to being easy to produce and administer, an effective oral vaccine would also have to have a built-in safeguard against digestion, particularly against the harsh acidic environments found in the stomach. The starchy endosperm in rice contains two types of protein storage organs, PB-I and PB-II, which are distinguished by their shape, density, and protein composition (29). The main storage proteins for PB-I are the alcohol-soluble prolamins (e.g., 13k prolamin) and the water-soluble glutelins (e.g., glutelin B1) (29, 30). Because they are water-soluble, the glutelins (PB-II) are more vulnerable to digestion in the gastrointestinal tract than are prolamins (PB-I). Immunoelectron microscopic analysis reveals that CTB is localized not only on the surface of PB-I, but also on the inside of PB-II (Fig. 2*A*). To examine the ability of the CTB accumulated in the rice PB to withstand protease digestion in the stomach, total seed proteins were subjected to pepsin treatment. Western blot analysis (Fig. 2*B*) revealed that the signal intensity of the 13k prolamin was not significantly changed by the pepsin treatment, whereas ≈90% of the glutelins were digested by pepsin under these conditions. In addition, ≈75% of the CTB accumulated in rice seed remained intact after pepsin treatment (Fig. 2*B*). These findings suggest that most

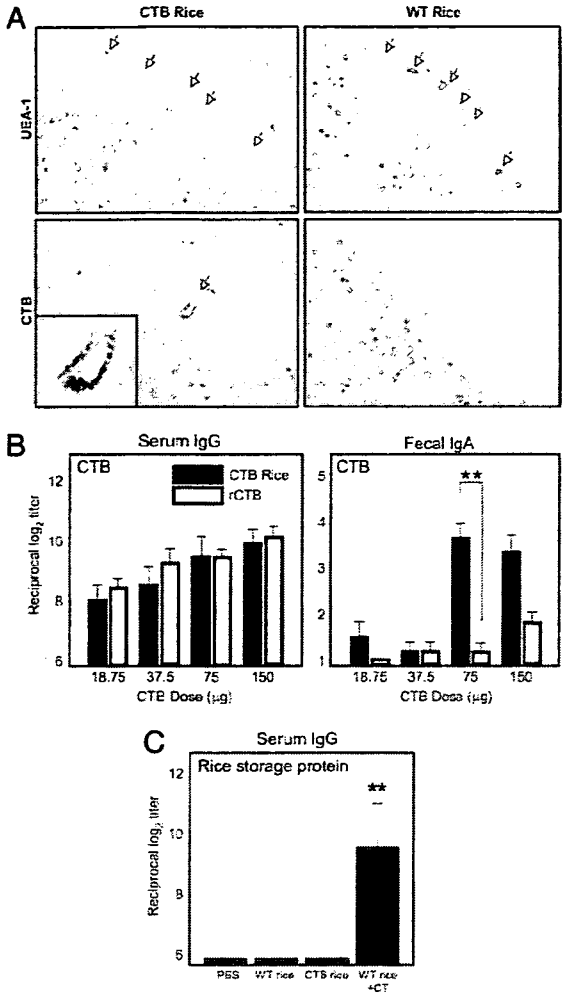

Fig. 3. Effective uptake of rice-expressed CTB by M cells for the induction of antigen-specific immune responses. (*A*) Rice-expressed CTB was administered into an intestinal loop containing PPs. Thirty minutes after the inoculation, the brisk CTBs were taken up by UEA-1-positive M cells (arrow), but not columnar epithelial cells. (*B*) When mice were orally immunized with rice-expressed CTB, purified rCTB, or nontransgenic rice dissolved in water or water alone as controls, equal levels of CTB-specific serum IgG responses were induced in mice immunized with rice-expressed CTB or purified rCTB, but not in mice receiving WT rice or water alone. In contrast, CTB-specific fecal IgA responses were also induced in mice immunized with a small amount (50 mg of seed powder containing 75 μg of CTB) of rice-expressed CTB, but not with an identical dose of purified rCTB. **, $P < 0.01$, CTB rice vs. rCTB. (*C*) Rice-expressed CTB did not induce rice storage protein-specific serum IgG responses. **, $P < 0.01$, WT rice plus CT vs. CTB rice.

of the CTB expressed in transgenic rice seed can be protected from the harsh conditions of the gastrointestinal tract. To characterize the mucosal immunogenicity of the rice-based oral vaccine in more detail, we opted to use the Kitaake CTB system for the remainder of the study.

Rice-Expressed CTB Is Effectively Taken Up by Antigen-Sampling M Cells for the Induction of Antigen-Specific Immune Responses. To confirm the M cell uptake of rice-expressed CTB, a suspension of rice-expressed CTB or nontransgenic WT rice was administered into the ligated small intestinal loops, including the PPs of naive mice. Histological analysis with *Ulex europaeus* agglutinin (UEA-1), which is a well known marker of murine M cells (31), demonstrated a strong presence of CTB antigen in UEA-1+ M cells (Fig. 3*A*). We

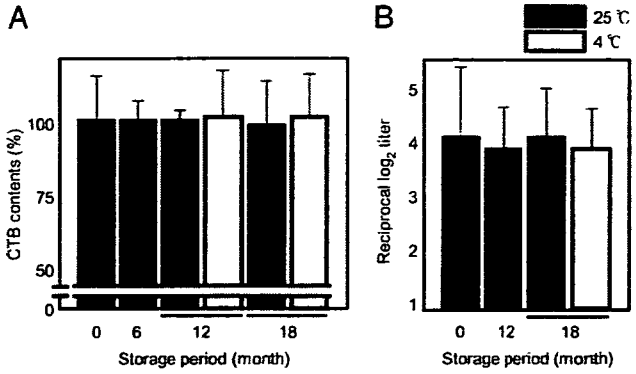

Fig. 4. Temperature stability of rice-expressed CTB. (*A*) One thousand rice seeds expressing CTB were preserved in a 500-ml sealed bottle for >1.5 years at 4°C as well as at RT (25°C). The content of CTB in preserved rice was not changed compared to that in freshly harvested rice (29 ± 4 μg per seed). (*B*) Mice were orally immunized with preserved rice-expressed CTB (50 mg of seed powder containing 75 μg of CTB) as described in Fig. 3. The preserved rice induced CTB-specific mucosal IgA responses that were comparable to those observed for freshly harvested rice.

next orally immunized mice with the seed powder of rice-expressed CTB or purified rCTB. Rice-expressed CTB induced CTB-specific serum IgG and fecal IgA antibodies (Fig. 3*B*). CTB-specific fecal IgA responses were also induced in mice immunized with a low dose of rice-expressed CTB (e.g., 50 mg of rice powder containing 75 μg CTB), whereas the same dose of purified rCTB induced no or very low levels of antigen-specific IgA responses (Fig. 3*B*). Furthermore, it should be emphasized that rice-expressed CTB induced no rice storage protein-specific immune responses (Fig. 3*C*). These findings demonstrated that the rice-based mucosal vaccine is an effective delivery vehicle for the induction of antigen-specific mucosal IgA responses.

Rice-Based Mucosal Vaccine Maintained Immunogenicity for More than 1.5 Years at RT. Inasmuch as our results provide supportive evidence for the protective advantage of rice-based mucosal vaccine, which includes stability in the harsh condition of the gastrointestinal tract (Fig. 2*B*), it was logical to examine whether the rice-based vaccine preserved at RT (25°C) for an extended period maintained its stability and mucosal immunogenicity. To this end, rice-based mucosal vaccine was preserved for 0.5, 1.0, or 1.5 years at either RT (25°C) or 4°C. Densitometry analysis revealed that the antigen in rice seed remained stable at RT for >1.5 years (Fig. 4*A*). Furthermore, the rice preserved at RT for 1.5 years induced the same level of CTB-specific fecal IgA responses as freshly harvested rice (Fig. 4*B*). These data suggest that the rice-based mucosal vaccine is more stable than the purified antigen of the subunit vaccine, as well as more effective for induction of IgA-committed mucosal immune responses.

Rice-Expressed CTB Induces Protective Immunity Against Cholera Toxin (CT). Finally, to examine the biological activities of antibodies induced by oral administration of rice-expressed CTB, CT-neutralizing activities were investigated by using a GM1-binding inhibition assay with GM1-ELISA (17) and an elongation assay with Chinese hamster ovary (CHO) cells (17, 32). When serum samples from mice orally immunized with rice-expressed CTB or WT rice were subjected to GM1-ELISA, the binding of CT to the coated GM1 ganglioside was blocked in the former but not the latter group of samples (Fig. 5*A*). The elongation assay also revealed no morphological changes in CHO cells cocultured with CT that had been pretreated with serum from mice orally vaccinated with rice-expressed CTB. In contrast, CT pretreated with

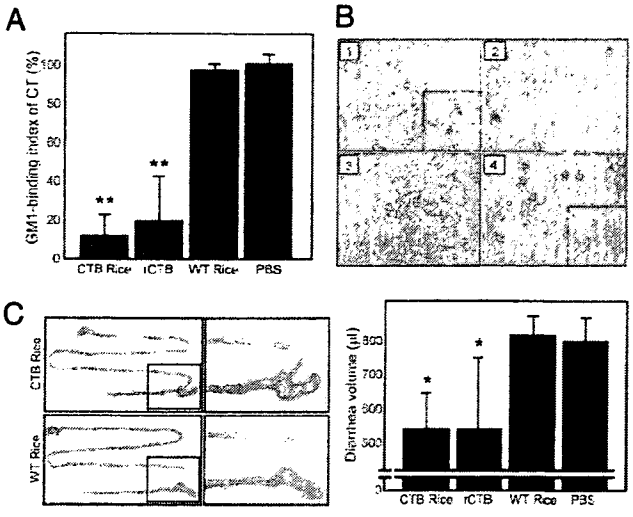

Fig. 5. Induction of protective immunity against CT by rice-expressed CTB. (*A*) The neutralizing index calculated with OD_{450} obtained by GM1-ELISA. The serum of mice immunized with rice-expressed CTB or purified rCTB, but not with nontransgenic rice or PBS, completely blocked the binding of CT to coated GM1-ganglioside. **, $P < 0.01$, CTB rice or rCTB vs. PBS. (*B*) The elongation assay with CHO cells revealed a morphology similar to normal cells (1) when the cells were stimulated with CT pretreated with serum from mice immunized with rice-expressed CTB (2) or purified rCTB (3), but a marked elongation when stimulated by CT pretreated with the serum from mice immunized with nontransgenic rice, PBS, or nontreated CT (4) as shown by the arrows. (*C*) In contrast to mice receiving WT rice or PBS, mice orally vaccinated with rice-expressed CTB showed no symptoms of diarrhea and a low level of intestinal water. *, $P < 0.05$, CTB rice or rCTB vs. PBS.

serum of mice immunized with WT rice showed a massive elongation of CHO cells (Fig. 5*B*) similar to that induced by the native form of CT. Most important, when orally challenged with CT, the mice vaccinated with rice-expressed CTB showed no clinical sign of diarrhea (Fig. 5*C*), whereas those fed the WT rice or PBS developed severe diarrhea. However, some mice immunized with purified rCTB suffered from diarrhea (Fig. 5*C*). Consistent with these findings, the volume of intestinal water in mice immunized with rice-expressed CTB was significantly lower after challenge with CT than in mice receiving WT rice or PBS (Fig. 5*C*). These data directly demonstrate that oral vaccination with rice-expressed CTB could offer a high degree of protection against CT challenge.

Discussion

In this study, we have developed a physically and chemically stable and immunologically effective vaccine antigen-expressing transgenic rice seed that can withstand the harsh environment of the gastrointestinal tract and induce protective immunity against mucosal infections. The use of transgenic rice for vaccine production offers several benefits over other plants for vaccine production. For the implementation of global vaccination strategy, a well designed oral vaccine system should satisfy the following criteria: (*i*) produce sufficient quantities of inserted antigen for the immunization (33), (*ii*) preserve the expressed antigen for a long time at RT (9, 34), (*iii*) induce protective immunity (8, 34), (*iv*) protect from enzymatic digestion in gastrointestinal tract (8), and (*v*) effectively deliver the inserted antigen to mucosal inductive tissues, including antigen-sampling M cells (8, 35). Although several plants have currently been used for the creation of an "edible vaccine," seed crops such as soybean, maize, wheat, or rice seem to be the most suitable plants for fulfillment of the previous requirements. It was recently shown that a soybean-based oral vaccine expressing heat-labile toxin B subunit (LTB) of *E. coli* induced antigen-specific IgG and IgA responses (36). Although maize also has been used for the expression of LTB (20), a biological nature of long-distance pollen scattering is the major environmental concern (37). Further, the difficulty of transforming the inserted gene by use of the wheat vector system unfortunately disqualified its suitability for the oral vaccine development. In contrast, rice self-fertilizes, and thus its pollen is considered to fly within only 10 m (37). In addition, rice plants have unique features in the storage of protein using two systems of PB-I and PB-II (29), which are suitable for accumulation of vaccine antigen. Furthermore, rice is the only crop that full of genome sequences was elucidated, and thus it easily applied the genetic information for the creation of gene-manipulated product (38). It is expected that this 430-Mb genome information contributes to the development of useful transgenic rice (38).

To show the unique features and feasibility of rice-based mucosal vaccine, we purposely used CTB as a vaccine antigen because CTB has been immunologically well characterized and extensively used for the analysis of antigen-specific immune response in both mucosal and systemic compartments. One of the major limitations of plant-based vaccines is the achievement of a high expression of inserted vaccine antigen that is sufficient to induce protective immunity (33). To achieve high expression and accumulation of inserted vaccine antigen in rice seed, an endosperm-specific expression promoter gene, 2.3-kb *GluB-1*, followed by an endoplasmic reticulum retention signal peptide, KDEL, was used for the expression of CTB (Fig. 1*A*). By optimizing the codon usage of CTB for expression in rice, the accumulation level of CTB was achieved at ≈2.1% of total seed protein (0.15% of seed weight) (Fig. 1 *E* and *F*). Although CTB has been expressed in the potato, the level of expression was ≈0.3% of total protein (0.002% of fresh weight) in potato tubers (17); ≈4% of total leave protein (0.5% of leave weight) was achieved in tobacco leaves by using a chloroplast expression system (35). However, the tobacco leaf is not applicable in the practical sense for oral vaccination. Thus, the use of the rice transgenic system allowed the efficient expression of inserted vaccine antigen, although we cannot directly compare the level of the inserted antigen expression to other previously published plant-based vaccine systems.

The SDS/PAGE and Western blot analyses with anti-CTB polyclonal antibody showed two protein species with 12 kDa, which was almost the same as that of authentic CTB (39), and 15 kDa were detected (Fig. 1 *E* and *F*), suggesting that part of CTB expressed in rice seed might contain a full or partial *GluB-1* signal peptide at the N terminus. The SDS/PAGE under nonreducing conditions and subsequent Western blot analyses showed that the molecular mass of two protein species was shifted to 55–65 kDa (Fig. 1*F*). Because these two protein species were recognized by anti-CTB polyclonal antibody and possessed a molecular weight comparable to a pentameric structure under natural condition, most rice-expressed CTBs were considered to be a functionally native pentameric form for the induction of an antigen-specific immune response.

The tolerableness of inserted vaccine antigen in rice seed against the harsh digestive tract environment was also attributed by the site of protein accumulation in the rice seed. In general, rice starchy endosperm cells contain two types of protein storage organelles (PB-I and PB-II) with a different shape, density, and protein composition (29). PB-I and PB-II mainly contain prolamins (e.g., 13k prolamin) and glutelins (e.g., glutelin B1) as storage proteins, which are defined as alcohol- and water-soluble proteins, respectively (29, 30). Thus, glutelins (PB-II) are considered to be more digestible and sensitive than prolamins (PB-I) in the gastrointestinal tract. The immunoelectron microscopic analysis showed that CTB accumulation occurred in PB-I and PB-II of the endosperm cells of the rice seed (Fig. 2*A*). Thus, the direction of inserted protein expression (e.g., CTB) in PB-I seems to be responsible for the tract of resistance against digestive enzyme activity, and thus allows for

the effectiveness in the induction of antigen-specific immune response by minimum dose of oral antigen. To support this view, an *in vitro* pepsin digestion study showed that most of prolamin and ≈75% of the CTB accumulated in the rice seed were protected from the pepsin treatment, whereas most of the glutelin in the rice seed and all purified rCTB were digested (Fig. 2*B*). In contrast, LTB expressed in maize kernel seems to be less resistant to peptic degradation when compared to CTB in the rice seed because LTB protein was accumulated mainly in the starch granules of transgenic maize kernels (40). These finding indicated that accumulation of vaccine antigen in PB-I would provide physicochemical stability against digestive enzymatic effects. Taken together, the rice-based vaccine was stable and more effective than the purified subunit vaccine, as well as other plant-based vaccines, in the harsh environment of the gastrointestinal tract for the induction of protective immunity.

As described above, in the gastrointestinal tract, the antigens ingested from the luminal site are taken up by PPs via antigen-sampling cells known as M cells for initiation of antigen-specific T helper cells and IgA-committed B cells (41). Targeting vaccine antigen delivery to M cells should be a goal in mucosal vaccine development. Intestinal loop assay with rice-expressed CTB demonstrated that CTB were taken up by M cells (Fig. 3*A*). According to many biodegradable microsphere studies, the <10-μm microspheres have been shown to be efficient delivery vehicles into antigen-sampling M cells in PPs (42). The diameters of rice PB-I and PB-II range in size between 1 to 2 μm and 3 to 4 μm, respectively (43). In addition, PB-I, but not PB-II, accumulated most of its CTB at the surface, perhaps further enhancing antigen uptake by M cells. Taken together, these results demonstrated that rice-expressed CTBs were not only effectively taken up by M cells located in the follicle-associated epithelium of PPs, but also could serve as effective carriers of mucosal vaccines to intestinal inductive tissues such as the PPs.

Oral immunization with rice-expressed CTB induced CTB-specific serum IgG and mucosal IgA responses (Fig. 3*B*). A low dose of rice-expressed CTB (e.g., 50 mg of rice powder containing 75 μg CTB) sufficiently induced CTB-specific mucosal IgA responses, whereas the same contents of purified rCTB induced no or low levels of antigen-specific IgA responses (Fig. 3*B*). The differences in required dosage may be because of the physicochemical stability exhibited by rice-based mucosal vaccines and their ability to withstand digestive effects. Although oral immunization with rice-expressed CTB can induce CTB-specific immune responses, it did not induce any rice storage protein-specific immune responses (Fig. 3*C*), suggesting that rice-expressed CTB did not show adjuvant activity for rice protein. Furthermore, Southern blot analysis confirmed the genetic stability of the CTB-transgenic rice (data not shown); CTB expressed in the rice seed could be preserved for >1.5 years not only at 4°C, but also at RT, without any degradation (Fig. 4*A*), and the rice preserved for 1.5 years at RT induced an equal level of CTB-specific fecal IgA responses as freshly harvested rice (Fig. 4*B*). Our results provided further evidence for a significant potential benefit of rice-based mucosal vaccine for the development of stable vaccine with immunogenicity. Thus, a rice-based mucosal vaccine can be introduced as a first cold-chain-free vaccine.

Finally, we showed the protective effect induced by oral immunization with a rice-based mucosal vaccine. CT binds its receptor GM1-ganglioside, which is ubiquitously expressed in intestinal epithelium, and causes severe diarrhea (39). Our results demonstrated that the serum from mice immunized with rice-expressed CTB completely blocked the binding of CT to GM1-ganglioside (Fig. 5*A*) and also inhibited the elongation of CHO cells caused by CT (Fig. 5*B*). Most important, mice immunized with rice-expressed CTB showed no clinical sign of diarrhea after orally challenged with CT, whereas some mice immunized with purified rCTB suffered from diarrhea (Fig. 5*C*) perhaps because the level of antigen-specific mucosal IgA was lower in the purified rCTB-fed group than in the group receiving rice-expressed CTB. Therefore, we conclude that the rice-based mucosal vaccine would be effective for the induction of protective immunity compared to other types of mucosal vaccine.

In summary, we have developed a rice-based oral vaccine that offers significant advantages over currently available vaccines. In the rice-based vaccine, the vaccine antigen, CTB, accumulated in the PBs of starchy endosperm cells, from which they were taken up by M cells for the induction of antigen-specific mucosal immune responses with neutralizing activity. In addition, the rice-based CTB vaccine remained stable and maintained immunogenicity at RT for >1.5 years and was protected from pepsin digestion *in vitro*. Taken together, these findings suggest that a rice-based oral vaccine would be a most effective and highly practical vaccine regimen against infectious diseases, whether naturally occurring or stemming from acts of bioterrorism. Given its cost effectiveness and ease of administration, it would be a vaccine whose benefits could be fully enjoyed in developing countries, where the need is often the greatest.

Materials and Methods

DNA Constructions and Transformation of Rice Plants. The CTB gene of *V. cholerae* was modified to a suitable form for rice seed and inserted into a binary vector (pGPTV-35S-HPT) (23). The resulting plasmid (Fig. 1*A*) was transformed in two lines of rice plants, *Oryza sativa* L. cv Kitaake (24) and Hosetsu (25, 26), using an *Agrobacterium*-mediated method described previously (27).

DNA and RNA Analyses. Using the cetyltrimethylammonium bromide extraction method, we extracted genomic DNA from the leaf tissues of transgenic rice and analyzed the integration of the CTB gene in genomic DNA using PCR (23). The expression of mRNA encoding CTB in the rice seed was analyzed by Northern blot as previously described (23). Briefly, total RNA (30 μg) extracted from the developing seeds of rice plants using the phenol/chloroform extraction method was separated on a 1.0% (wt/vol) formaldehyde/agarose gel and transferred to Hybond N$^+$ membranes (GE Healthcare, Piscataway, NJ). The amplified CTB gene was used as a hybridization probe after labeling with [α-^{32}P] dCTP (GE Healthcare).

Protein Analyses. Total seed protein was extracted from seeds as described previously (23). Briefly, seeds of rice plants were ground to a fine powder using a Multibeads shocker (Yasui Kikai, Osaka, Japan) and extracted in 2% (wt/vol) SDS, 8 M urea, 5% (wt/vol) β-mercaptoethanol, 50 mM Tris·HCl (pH 6.8), and 20% (wt/vol) glycerol before being separated by SDS/PAGE with a 15% to 25% gradient polyacrylamide gel (Daiichi Pure Chemical, Tokyo, Japan). The gel was subsequently transferred to Hybond-P PVDF membranes (GE Healthcare) for Western blot analysis with 5 μg/ml rabbit anti-CTB antibody prepared in our laboratory. Accumulation levels of CTB were determined by densitometry analysis of Western blot against a standard curve generated with the use of rCTB expressed in *Bacillus brevis* and purified by using immobilized galactose (Pierce, Rockford, IL) in our laboratory (44). Using immunoelectron microscopic analysis, the distribution of CTB expressed in rice seed was analyzed. Briefly, rice seeds (at 12–15 days after flowering) were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and 0.1% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) and embedded in LR White (London Resin, Hampshire, U.K.). Ultrathin sections (150 nm) were stained with 5 μg/ml rabbit anti-CTB antibody, followed by gold particle (20 nm)-conjugated goat anti-rabbit IgG (E.Y. Labs, San Mateo, CA) diluted to 1:10. The sections were then stained with 4% uranyl acetate and examined by using a transmission electron microscope (JEM100S; JEOL, Tokyo, Japan).

Pepsin Treatment. Seed powder (10 mg containing 15 μg of CTB) and purified rCTB (15 μg) were incubated with 0.5 mg/ml pepsin (2,500–3,500 units per mg protein; Sigma–Aldrich, St Louis, MO) in 0.1 ml of 0.1 M sodium acetate buffer (pH 1.7) with gentle rocking for 1 h at 37°C. After neutralization, the degradation of CTB, glutelin, or prolamin was analyzed by Western blot with 5 μg/ml rabbit anti-CTB, anti-glutelin GluB-1, or anti-13k prolamin antibodies prepared in our laboratory, respectively.

Uptake of CTB by M cells. A rice-expressed CTB or a nontransgenic rice was administered into an intestinal loop (≈1 cm) containing PPs of mice anesthetized by using 2 mg of ketamine (Sigma–Aldrich) per mouse. Thirty minutes after inoculation, the tissues were removed and fixed in tissue fixative (Genostaff, Tokyo, Japan) overnight at 4°C and then embedded in paraffin. Several mirror sections (5 μm) were subjected to immunohistochemistry with 5 μg/ml anti-CTB antibody and biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and to lectin histochemistry with 20 μg/ml biotinylated UEA-1 (Vector Laboratories). Both sections were finally incubated with SAB-PO (Nichirei, Tokyo, Japan) and visualized for the distribution of CTB and the localization of M cells by 3,3′-diaminobenzidine tetrahydrochloride (Dojin Laboratories, Kumamoto, Japan).

Oral Immunization. One immunization study was carried out by using 6-week-old C57BL/6J and BALB/c mice (CLEA, Tokyo, Japan). On six occasions at 2-week intervals, mice (six mice per group) were orally immunized with 12.5, 25, 50, or 100 mg of CTB-transgenic rice, with a corresponding dose of purified rCTB (18.75, 37.5, 75, or 150 μg) or with either 100 mg of nontransgenic rice dissolved in water or water alone as controls. For examination of the adjuvant effect of rice-expressed CTB, 100 mg of CTB-transgenic or WT rice was orally immunized with or without 10 μg of CT (List Biological Laboratories, Campbell, CA). One week after the final immunization, serum and fecal extracts were collected, and CTB or rice storage protein-specific Ig responses were measured by ELISA with 5 μg/ml rCTB or 20 μg/ml rice-storage protein extracted with 0.01% Triton X-100 as described previously (45).

Neutralizing Assay. Serial-diluted serum collected from immunized mice were treated with 50 ng/ml of CT and subjected to GM1-ELISA as previously described with some modifications (17). Briefly, 5 μg/ml of monosialoganglioside GM1 (Sigma–Aldrich)-coated 96-well plates (Thermo, Milford, MA) were incubated with CT that had been treated first with serum from immunized mice and then with an HRP-conjugated anti-CTB antibody prepared in our laboratory. The color was developed with the addition of TMB substrate (Moss, Pasadena, MD), and absorbance was measured at 450 nm. In addition, a CHO cell (ATCC, CCL-61) assay (32) was performed by using serum treated with 50 ng/ml CT. After 14 h of stimulation in 5% CO_2 in a humidified incubator at 37°C, morphological changes were observed under a microscope. In addition, we performed an *in vivo* challenge experiment with CT. The vaccinated mice were orally challenged with 20 μg of CT. After 14 h, clinical symptoms of diarrhea were observed, and the volume of intestinal water was measured.

Data Analysis. Data are expressed as the mean ± SD. All analyses for statistically significant differences were performed with Tukey's *t* test, with *P* values of <0.01 and <0.05 considered significant.

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Selective suppression of T_h2-mediated airway eosinophil infiltration by low-molecular weight CCR3 antagonists

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Abstract

The effects of selective CC chemokine receptor (CCR)-3 antagonists on antigen-induced leukocyte accumulation in the lungs of mice adoptively transferred with *in vitro*-differentiated T_h1 and T_h2 were investigated. Inhalation of antigen by mice injected with T_h1 and T_h2 initiated the migration of T cells themselves into the lungs. Subsequently, neutrophils massively accumulated in T_h1-transferred mice, whereas eosinophil infiltration was specifically induced by T_h2. CCR3 antagonists, SB-297006 and/or SB-328437, suppressed antigen-induced accumulation of T_h2 as well as eosinophils in the lungs, whereas they failed to affect T_h1-mediated airway inflammation. Not only T_h2 and eosinophil infiltration but also cellular mobilization in T_h1-transferred mice was attenuated by an anti-CC chemokine ligand-11 antibody. CCR3 antagonists reduced chemokine production in the lungs of mice transferred with T_h2 but not T_h1, suggesting that down-regulation of chemokine synthesis is involved in the selective inhibition of T_h2-mediated eosinophil infiltration by CCR3 antagonists.

Introduction

The crucial contribution of T_h2 to the development of allergic eosinophilic inflammation has been established (1). Accumulating evidence suggests that CC chemokine receptor (CCR)-3 plays a critical role in the accumulation of eosinophils as well as T cells during allergic inflammation (2–4). Enhanced expression of CCR3 and its ligands has been recognized in bronchial asthma (5). Gene disruption and/or blockade of CCR3 as well as its ligand, CC chemokine ligand (CCL)-11, resulted in a reduction of eosinophilic infiltration in animal models of asthma (6–9).

However, the contribution of CCR3 to allergic diseases remains controversial in view of the apparently contradictory findings that substantial eosinophilic inflammation still occurs in animal models of asthma under disruption or neutralization of CCR3 as well as CCL11 (6–10). Furthermore, Yang *et al.* (11) found no difference in the recruitment of eosinophils between CCL11-intact and -deficient mice, using several models of inflammation. Humbles *et al.* (9) demonstrated up-regulation of antigen-induced bronchial hyperresponsiveness (BHR) in CCR3-knockout mice. Above all, the lack of a selective antagonist against CCR3 that is available for *in vivo* administration has confounded accurate assessment of the clinical efficacy of CCR3 blockers to treat allergic disorders. A number of CCR3 antagonists have been investigated (2), though most of them are peptides/proteins. Recently, the potent, selective, low-molecular weight CCR3 antagonists, SB-297006 and SB-328437, were identified (12). These compounds inhibit CCL11-induced Ca^{2+} mobilization and eosinophil chemotaxis *in vitro*. The selectivity of these compounds was confirmed using panels of chemokines and other seven-transmembrane receptors (12).

In order to delineate the role of CCR3 in the development of allergic inflammation in more detail, an antigen-induced murine airway inflammation model, separately initiated by T_h1 and T_h2, was employed in this study, with monitoring of the migration of inflammatory cells including antigen-specific

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T cells. The contribution of CCR3 and its ligands to T_h1- and T_h2-mediated cellular mobilization in the lungs and BHR was examined using these selective low-molecular weight antagonists and blocking antibodies.

Methods

In vitro polarization of T cells

Ovalbumin (OVA)-specific naive $CD4^+$ T cells were isolated from the spleen of mice expressing the transgene for DO11.10 TCR$\alpha\beta$ by a combination of negative and positive selection using a $CD4^+$ T cell isolation kit and CD62L microbeads, respectively, with a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, Germany). Cells were cultured in AIM-V medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) in the presence of 100 $\mu g\ ml^{-1}$ OVA, 10 $U\ ml^{-1}$ recombinant IL-2 (BD Biosciences, San Jose, CA, USA) and X-ray-irradiated splenocytes of BALB/c mice. For T_h1 phenotype development, 10 $U\ ml^{-1}$ recombinant murine IL-12 (PeproTech, Rocky Hill, NJ, USA) and 1 $\mu g\ ml^{-1}$ neutralizing anti-IL-4 mAb (BD Biosciences) were added and for T_h2 phenotype development, 10 $U\ ml^{-1}$ recombinant murine IL-4 (PeproTech) and 1 $\mu g\ ml^{-1}$ anti-IL-12 mAb (BD Biosciences) were used. Seven to ten days after stimulation, cells were harvested, purified by centrifugation over Ficoll-Paque (GE Healthcare Bio-Sciences, Uppsala, Sweden) and then used as T_h1 and T_h2. To determine the integrity of polarization, cells were activated by plate-bound anti-CD3 mAb (145-2C11; BD Biosciences) for 24 h at 37°C in a 5% CO_2-humidified incubator. For antibody immobilization, wells of culture plates were pre-incubated with 10 $\mu g\ ml^{-1}$ anti-CD3 mAb in 0.05 M carbonate–bicarbonate buffer (pH 9.6) at 4°C overnight. The resulting culture supernatants were collected and subjected to measurement of cytokine and chemokine levels by ELISA.

CCR expression in T cells

Naive and differentiated T cells were re-suspended ($10^6\ ml^{-1}$) in AIM-V medium containing 10% FBS and either left unstimulated or stimulated with plate-bound anti-CD3 mAb. After 8 h, cells were harvested and total RNA was then extracted and reverse transcribed using oligo(dT)12–18 primer and ReverTra ACE® (Toyobo, Osaka, Japan). Quantitative real-time reverse transcription (RT)–PCR was performed using Assay-on-Demand™ Gene Expression products (TaqMan® MGB probes) with an ABI prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). Transcripts of CCR3, CCR4, CCR5, CCR8 and CXC chemokine receptor (CXCR)-3 were normalized to 18S rRNA abundance. In some experiments, the expression of CCR3 on the surface of T_h2 was determined by flow cytometry upon staining with an anti-CCR3 antibody (R&D systems) or isotype-matched control antibody.

Chemotaxis assay

Chemotaxis of T_h2 was measured using 24 transwells with polycarbonate membranes (3 μm; Corning Incorporated, Corning, NY, USA). Chemokines were diluted in 0.6 ml chemotaxis buffer (RPMI 1640 medium containing 0.5% BSA) and added to the lower chemotaxis chamber. T_h2 (10^6) were re-suspended in 0.1 ml chemotaxis buffer and added to the top chamber insert. Chemotaxis plates were then incubated for 2 h. After incubation, the cells from the bottom well were collected and counted by flow cytometry.

Cell transfer and challenge procedures

Before transfer, polarized T_h1 and T_h2 were stained with a fluorescein-based dye, 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR, USA), as described previously (13). Twenty-four hours after cell transfer, mice were challenged with aerosolized 10% OVA dissolved in 0.9% saline. In some experiments, the same challenge was performed 1 week after the first challenge. For blocking studies, SB-297006 [(*S*)-ethyl-2-benzoylamino-3-(4-nitrophenyl)propionate] or SB-328437 [(*S*)-methyl-2-napthoylamino-3-(4-nitrophenyl)propionate], synthesized in the Discovery Research Laboratory of Tanabe Seiyaku Co., Ltd (Osaka, Japan), suspended in PBS containing 0.1% Tween 80, was administered subcutaneously. The dose of the compounds was determined as 100 $mg\ kg^{-1}$, since both agents displayed a slight sedative effect in mice that received more than this dose (data not shown). A rabbit anti-CCL7 or anti-CCL11 antibody (PeproTech) dissolved in PBS was administered intravenously 30 min before OVA challenge. The vehicle alone did not affect any parameter examined (data not shown). Cellular infiltration in the lungs was examined by bronchoalveolar lavage (BAL) as described previously (13, 14). BHR was assessed as the degree of bronchoconstriction following infusion of 300 $\mu g\ kg^{-1}$ acetylcholine, at which the maximum difference in respiratory overflow volume between specific antigen- and BSA-challenged animals was obtained, according to the method described previously (14). We have demonstrated that the cellular profile in BAL fluid well reflects the pathological features and resultant BHR developed in the lungs of T_h2-transferred mice (13, 14).

Cytokine ELISA

Purified rat anti-mouse IL-5 mAb (TRFK-5; BD Biosciences) and anti-mouse CCL7 antibody (R&D Systems) were used as the capture antibodies and biotinylated rat anti-mouse IL-5 mAb (TRFK4; BD Biosciences) and goat anti-mouse CCL7 antibody (R&D Systems) as the detection antibodies for ELISA. IL-4 and IFN-γ were assayed using Duo Set® (Genzyme, Cambridge, MA, USA), CCL2 using OptEIA™ mouse MCP-1 set (BD Biosciences) and CCL11 using a Quantikine® M mouse eotaxin immunoassay kit (R&D Systems), according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using one-way analysis of variance with Bonferroni's method. $P < 0.05$ was considered to indicate statistical significance.

Results

Characterization of in vitro-differentiated T cells

The first experiment was carried out to verify successful polarization of DO11.10 T cells after stimulation culture under