

T-box 21 transcription factor is responsible for distorted T_H2 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 Tokyo, Tsukuba, and Sagamihara, Japan

Background: Regardless of T_H1/T_H2 theory, CD4⁺ T cells of patients with allergic asthma, a typical T_H2 disease, and those of healthy subjects expressed equivalent levels of IFN- γ , even though T_H2 cytokines were significantly upregulated in asthmatic patients.

Objective: The mechanisms underlying distorted T_H2 cell polarization in human T cells were elucidated.

Methods: Cytokine-producing activity and the expression of T_H1/T_H2-specific transcription factors in naive, T_H1/T_H2, or both CD4⁺ T cells derived from human peripheral and cord blood were comparatively analyzed. The mechanisms of the differential expression of T-box 21 transcription factor (T-bet) in the cells were assessed by determining the chromatin accessibility at the *TBX21* gene. The functional roles of T-bet and other transcription factors in human T_H1/T_H2 differentiation were further investigated.

Results: T_H2 cells derived from naive CD4⁺ T cells in peripheral blood but not in cord blood produced IFN- γ . T-bet was expressed in peripheral, but not cord blood, resting naive T cells. Consistently, the accessibility at the proximal *TBX21* gene promoter in peripheral naive T cells was higher than that in cord blood naive T cells. IFN- γ -producing activity was induced in T_H2-differentiated cord blood T cells by means of ectopic expression of T-bet. In addition, a reduction of T-bet in peripheral T cells suppressed IFN- γ production. T-bet not only upregulated IFN- γ but also downregulated IL-4 and IL-13 gene transcription, independently of the modification of T_H1/T_H2 balance.

Conclusion: The expression of T-bet at a naive stage is crucial for the development of IFN- γ -producing T cells in human peripheral blood, even in T_H2-related diseases. (J Allergy Clin Immunol 2009;123:813-23.)

Key words: Allergy, bronchial asthma, cord blood, IFN- γ , IL-4, human T cells, T-bet, T_H1/T_H2

CD4⁺ T cells play a central role in immune responses. Among the at least 4 currently recognized, distinct CD4⁺ T-cell subsets (ie, T_H1, T_H2, T_H17, and regulatory T cells), T_H1 and T_H2 subpopulations were identified about 2 decades ago and have been extensively investigated thus far.^{1,2} T_H1 cells specifically produce IFN- γ , which controls cellular immunity, whereas T_H2 cells preferentially secrete IL-4, IL-5, and IL-13, which regulate humoral immunity.^{2,3} Impairment of the T_H1/T_H2 balance has been suggested to cause a variety of diseases. For example, acceleration of T_H1 response is recognized in autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, whereas excessive T_H2 responses are observed in allergic diseases, including allergic asthma and atopic dermatitis.^{2,3} This T_H1/T_H2 paradigm has led to the concept that immunotherapy converting T_H2 into T_H1 responses should be effective for the treatment of allergic diseases.⁴

However, the true nature of T-cell cytokine responses, especially in human diseases, is more complex than can be explained by T_H1/T_H2 dichotomy.⁵ For example, bronchial biopsy specimens from asthmatic patients are characterized by CD4⁺ T cells displaying T_H2 cytokines,⁶ suggesting that allergic asthma is considered a representative T_H2 disease. Nevertheless, although there might be a reduced presence of T_H1 cytokines,^{7,8} allergic asthma is also associated with the presence of IFN- γ . IFN- γ can readily be identified both as mRNA and protein in bronchoalveolar lavage fluid and biopsy specimens.⁶ ten Hacken et al⁹ showed that increased serum IFN- γ levels correlated with airway hyperresponsiveness in asthmatic patients. In agreement with the observation that the asthmatic lung already contains a significant amount of IFN- γ , a clinical trial of IFN- γ , at least in nebulized form, failed to demonstrate the efficacy of this cytokine for the treatment of allergic asthma.¹⁰ Therefore unlike murine cells, human CD4⁺ T cells might be programmed to produce IFN- γ , even if they are differentiated into T_H2 cells.

Although the reason for the inconsistency of the T_H1/T_H2 paradigm in human CD4⁺ T cells has been poorly elucidated, neonates are known to display immature immunity to infections, partly because of poor T_H1 responses.^{11,12} It has been demonstrated that the IFN- γ -producing activity of T cells in umbilical cord blood was weaker than that in adult peripheral blood.¹³⁻¹⁵ In addition, differential expression of several intracellular signaling molecules, including T_H1- and T_H2-specific transcription factors in cord blood and adult T cells, has been observed.^{12,16,17} Therefore detailed and comparative analysis of peripheral and cord blood CD4⁺ T cells is likely to be helpful for understanding the mechanisms of the distorted T_H2 differentiation in human T_H2-related diseases.

From ^athe Department of Allergy and Immunology and ^bthe Cytokine Project, Tokyo Metropolitan Institute of Medical Science, Tokyo; ^cthe Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN, Tsukuba Institute, Tsukuba Ibaraki; ^dthe Department of Allergy and Rheumatology, Tokyo Metropolitan Komagome Hospital, Tokyo; and ^ethe Department of Obstetrics and Gynecology and ^fthe Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Sagamihara, Kanagawa.

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Reprint requests: Osamu Kaminuma, DVM, PhD, Department of Allergy and Immunology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan. E-mail: kaminuma-os@igakuken.or.jp.

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Abbreviations used

EGFP: Enhanced green fluorescent protein
 FITC: Fluorescein isothiocyanate
 PE: Phycoerythrin
 REA: Restriction enzyme accessibility
 shRNA: Small hairpin RNA
 STAT4: Signal transducer and activator of transcription 4
 VSV-G: Vesicular stomatitis virus G glycoprotein

In accordance with previous findings, we here investigated the increased expression of T_H2 cytokines but not the downregulation of IFN- γ in $CD4^+$ T cells of patients with allergic asthma. The significant IFN- γ -producing property, even under T_H2 conditions, was specifically induced in peripheral, but not cord blood, $CD4^+$ T cells. The mechanisms of the difference between peripheral and cord blood cells were analyzed in view of the expression of T_H1/T_H2 -specific transcription factors by means of Western blotting and restriction enzyme accessibility (REA) assay. A crucial role of T-box transcription factor (T-bet) in T_H1/T_H2 differentiation was demonstrated in overexpression and knockdown studies by using a lentivirus infection system, real-time RT-PCR, and promoter reporter assay.

METHODS**Subjects**

This study was performed according to the Declaration of Helsinki principles and was approved by the Ethics Committee of the Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Komagome Hospital (Tokyo Japan), and Sagami National Hospital (Kanagawa, Japan). Thirty-seven adult patients referred to the outpatient clinic in these hospitals (17 male and 20 female patients; age [mean $\pm$ SD], 47.9 ± 18.9 years) were included in this study. All subjects met the criteria of mild-to-moderate asthma according to the "International consensus report on diagnosis and management of asthma" and demonstrated at least a 20% improvement in FEV_{1.0} after β_2 -agonist inhalation. The total serum IgE level was 652 ± 728 IU/mL. Treatment with β_2 -agonists, anticholinergics, and theophylline was stopped at least 12 hours before blood sampling. Patients receiving corticosteroids were excluded from the study. Human umbilical cord blood was collected at the time of elective cesarean section or normal spontaneous delivery of nonallergic healthy mothers. Written informed consent was obtained from all subjects, including 18 healthy volunteers (10 male and 8 female subjects; age, 62.4 ± 12.3 years), before investigations.

Plasmid constructs

The PCR fragment of cDNA encoding full-length human T-bet (amino acids 1-533), GATA-3 (amino acids 1-443), c-Maf (amino acids 1-373), and signal transducer and activator of transcription 4 (STAT4; amino acids 1-748) was subcloned in frame into an appropriate site in the CMV promoter-driven lentivirus expression vector combined with an internal ribosomal entry site sequence followed by the coding sequence of a luminescent protein, Venus, at the C-terminal end.^{18,19} In some experiments the SV40 promoter site in a pMACS-kk.II expression vector (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was replaced with the CMV promoter plus or minus the T-bet cDNA-coding sequence (pMACS-T-bet). T-bet was also subcloned into the pEF6/His vector (pEF-T-bet; Invitrogen, Carlsbad, Calif). To construct a small hairpin RNA (shRNA) expression vector, the human phosphoglycerate kinase eukaryotic promoter-driven puromycin resistance gene in a Mission shRNA clone containing sense and antisense 21-bp shRNA sequences derived from human T-bet cDNA (sense 5'-CGCTTCCACACGCATATCTT-3') and its nontarget shRNA control

vector (Sigma-Aldrich, St Louis, Mo) were replaced by human elongation factor 1 α promoter-driven Venus expression cassette.^{19,20} The human *IL4* promoter (-1105 to +4) and *IL13* promoter (-1077 to +49) were subcloned into the phosphorylated enhanced green fluorescent protein (EGFP) 1 vector (IL-4-EGFP and IL-13-EGFP; BD Biosciences, San Jose, Calif). The human IFN γ promoter-reporter vector (IFN- γ -EGFP) was described previously.²¹ The correct sequences of all constructs were verified by means of sequencing.

Preparation of lentivirus-expressing cDNA and shRNA

Lentiviruses pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G) were prepared as described previously, with slight modifications.^{18,20} Briefly, 293T cells were transfected with 3 plasmids: packaging construct (pCAG-HIVgp), VSV-G and Rev-expressing construct (pCMV-VSV-G-RSV-Rev), and lentivirus vectors, as described above. The resulting culture supernatant was concentrated by means of ultracentrifugation, and the virus pellet was resuspended in Hank's balanced salt solution. Titers of virus stocks were determined as described previously.²⁰

Cells, lentivirus infection, and transfection

$CD4^+$ T cells were purified from peripheral and cord blood mononuclear cells by means of positive selection with $CD4$ microbeads with a magnetic cell sorting system (Miltenyi Biotec). The purity of resulting cells was greater than 95%, as determined by means of flow cytometry. $CD4^+CD45RO^-$ naive T cells were also purified by using a combination of negative and positive selection with $CD45RO$ and $CD4$ microbeads, respectively. The resulting cells were analyzed for the expression of cell-surface molecules by means of staining with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti- $CD25$, anti- $CD44$, anti- $CD62L$, anti- $CD69$, anti- $CD127$ (eBioscience, San Diego, Calif), anti-CCR7, anti-IL-4 receptor α , anti-IL-12 receptor $\beta 1$ (R&D Systems, Inc, Minneapolis, Minn), and anti-IL-12 receptor $\beta 2$ (BD Bioscience) antibodies. Naive T cells were cultured in AIM-V medium (Invitrogen) in the presence of 0.01 μ g/mL anti- $CD3$ antibody (OKT3; Ortho Biotech, Bridgewater, NJ), 1 μ g/mL anti- $CD28$ antibody (BD Biosciences), and 10 U/mL recombinant IL-2 (Shionogi, Osaka, Japan). For T_H1 phenotype development, 100 ng/mL recombinant IL-12 (Peprotech, Rocky Hill, NJ) and 40 μ g/mL anti-IL-4 antibody (eBioscience) were added at the start of culture, and for T_H2 development, 400 ng/mL recombinant IL-4 (Peprotech) and 40 μ g/mL anti-IL-12 and anti-IFN- γ antibodies (eBioscience) were used. In neutralizing conditions no other recombinant cytokine or anti-cytokine antibody than IL-2 was added. For protein overexpression and knockdown study, cells were infected with viral stock solutions at a multiplicity of infection of 50 to 200 at 24 hours after the start of culture. After 7 to 10 days of stimulation, cells were harvested, purified by means of centrifugation over Ficoll-Paque, and then used as T_H1/T_H2 -polarized or neutrally developed cells. In some experiments lentivirus infection-positive cells were purified with a FACS Aria cell sorting system (BD Biosciences).

Simian virus 40 T antigen-transfected human leukemic Jurkat T (Jurkat Tag) cells were transfected with various amounts of plasmid DNAs by means of electroporation, as described previously.²² In some experiments transfection-positive cells were purified by means of positive selection by using H-2k microbeads with a magnetic cell sorting system. The purity of resulting cells was greater than 95%, as determined by means of flow cytometry with anti-H-2k-FITC antibody.

Immunoprecipitation and immunoblotting

Whole cell lysates prepared from 10^7 cells, as described previously,²² were incubated with 5 μ g of anti-T-bet (4B10) antibody for 2 hours, followed by the addition of 40 μ g of protein G-Sepharose beads for an additional 2 hours at 4°C. The beads were then washed 4 times, boiled in 20 μ L of Laemmli buffer, and subjected to SDS-PAGE. Immunoblotting against endogenous protein in whole cell lysates, immunoprecipitation samples, or both was performed by

using anti-T-bet (4B10), anti-GATA-3 (HG3-31), anti-c-Maf (M-153), anti-STAT4 (C-20), anti-actin (C-11; Santa Cruz Biotechnology, Santa Cruz, Calif), and anti-phosphotyrosine (PY-20; BD Bioscience) antibodies, as previously described.²¹

Intracellular cytokine staining

After stimulation culture, CD4⁺ T cells were restimulated with 5 nmol/L phorbol 12-myristate 13-acetate plus 1 μ mol/L ionomycin for 6 hours at 37°C in the presence of 2 μ mol/L 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-4 (MP4-25D2)-FITC or anti-IL-4-PE (eBioscience), anti-IFN- γ (4S.B3)-PE (BD Biosciences), anti-GATA-3-PE (BD Bioscience), and anti-T-bet-Alexa647 (eBioscience) antibodies. Flow cytometric analysis was performed on a FACScalibur with CellQuest software (BD Biosciences).

mRNA expression

Total RNA was extracted from freshly isolated CD4⁺ T cells or cells stimulated with or without 2.5 μ g/mL anti-CD3 antibody plus 1 μ g/mL anti-CD28 antibody in the presence of 5 μ g/mL anti-mouse IgG crosslinking antibody or 5 nmol/L phorbol 12-myristate 13-acetate plus ionomycin for 6 hours at 37°C. After reverse transcription with oligo(dT)12-18 primer and ReverTra ACE (Toyobo, Osaka, Japan), quantitative real-time RT-PCR for IL-4, IL-5, IL-13, IFN- γ , GATA-3, c-Maf, STAT4, and T-bet was performed by using Assay-on-Demand Gene Expression Products (TaqMan MGB probes), with specific probes listed in Table E1 in this article's Online Repository at www.jacionline.org, and an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, Calif), as described previously.²¹

Reporter assay and ELISA

After 16 hours of stimulation, the promoter-enhancer activity was assessed as fluorescence of synthetic EGFP detected by means of flow cytometry, as described previously.²¹ In some experiments the concentrations of IL-4, IL-13, and IFN- γ in the culture supernatant were measured by means of ELISA with READY-SET-GO ELISA sets (eBioscience), according to the manufacturer's instructions.

REA assay

REA assays were performed according to the method of Guo et al,²³ with modifications. Briefly, 1×10^6 cells were washed twice in cold PBS and resuspended in lysis buffer (60 mmol/L KCL, 15 mmol/L NaCl, 5 mmol/L MgCl₂, 10 mmol/L Tris-HCl [pH 7.5], 300 mmol/L sucrose, and 0.625% Nonidet P-40) with freshly supplied protease inhibitors. After cells were lysed on ice for 10 minutes, the nuclei were placed in pellets at 2000 rpm for 10 minutes at 4°C. Nuclei were then treated with varying amounts of *Hae*III digestion buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 7.9], 10 mmol/L MgCl₂, and 1 mmol/L dithiothreitol) at 37°C for 24 hours. The reactions were stopped by means of addition of EDTA to a final concentration of 10 mmol/L. Proteinase K and SDS were added to final concentrations of 0.1 mg/mL and 1%, respectively, and the samples were incubated at 55°C for a minimum of 4 hours. The samples were extracted once with saturated phenol and twice with phenol-chloroform. The DNA was then precipitated with ethanol. Purified DNA was subjected to quantitative real-time RT-PCR by using TaqMan Genomic-Assays (MGB probes) with an ABI Prism 7900 sequence detection system. The 5' and 3' primers and specific probe sets used to detect the digestion of the *TBX21* gene are described in Table E2 in this article's Online Repository at www.jacionline.org. The results were expressed as the relative amount of intact DNA in comparison with non-enzyme-treated control values.

RESULTS

CD4⁺ T cells are distortedly differentiated into T_H2 cells in allergic asthma

Regardless of the T_H1/T_H2 theory, it has not been clearly evaluated whether T_H1 cytokines are downregulated in human T_H2-related diseases. Therefore the expression of T_H1/T_H2 cytokines and transcription factors in CD4⁺ T cells of patients with allergic asthma, a typical T_H2 disease, was first investigated. mRNA expression levels in freshly isolated CD4⁺ T cells from peripheral blood were measured by means of quantitative real-time RT-PCR to exclude the difference in the antigen specificity among individual patients. Consistent with previous reports,^{3,4} IL-4, IL-5, and IL-13 expressions in asthmatic patients were significantly higher than those in healthy subjects. However, the same level of IFN- γ expression was observed in both groups (Fig 1). In addition, there was no significant difference in the expression of the T_H1-specific transcription factors STAT4 and T-bet and the T_H2 transcription factors GATA-3 and c-Maf. These findings suggest that human CD4⁺ T cells are not differentiated into typical T_H2 cells, even in T_H2-related diseases.

Human peripheral naive CD4⁺ T cells-derived T_H2 cells produce IFN- γ

The potential for T_H1/T_H2 differentiation of peripheral naive CD4⁺ T cells was next investigated to clarify the mechanisms underlying the lack of downregulation of IFN- γ in allergic asthma. In this experiment naive CD4⁺ T cells prepared from cord blood were also used because it has been suggested that cord blood T cells display poor IFN- γ -producing activity.¹³⁻¹⁵ As shown in Fig 2, A, peripheral blood and cord blood mononuclear cells contained approximately 9% and 25% CD45RA⁺ naive CD4⁺ T cells, respectively. From both types of mononuclear cells, CD4⁺CD45RA⁺ T cells were successfully enriched (approximately 99% purity) after a combination of negative and positive selection. The resulting peripheral and cord blood CD4⁺CD45RA⁺ cells were composed of a CD25⁻CD44⁺CD62L⁺CD69⁻CD127⁺CCR7⁺ homogeneous population (Fig 2, B), even though the expression levels of the positive molecules differed in individual subjects (data not shown), suggesting that these cells are recognized as typical naive T cells. IL-4 receptor α and IL-12 receptor β 1, but not IL-12 receptor β 2, were similarly expressed on both types of cells. After stimulation culture under T_H1 and T_H2 conditions, respective phenotypes were clearly induced from cord blood naive CD4⁺ T cells (Fig 2, C). T_H1 differentiation was also successfully induced, although peculiar T_H2 differentiation occurred in peripheral blood naive CD4⁺ T cells. Thus IFN- γ -producing cells were significantly developed, even under T_H2-skewing conditions (Fig 2, C).

Peripheral naive CD4⁺ T cells significantly express T-bet because of high accessibility of the proximal promoter region in the *TBX21* gene

The expression of T_H1- and T_H2-specific transcription factors in peripheral and cord blood naive/T_H1/T_H2 cells was comparatively analyzed by means of Western blotting to elucidate the reason for the distorted T_H2 differentiation in human peripheral CD4⁺ T cells. As shown in Fig 3, A, GATA-3 expression was

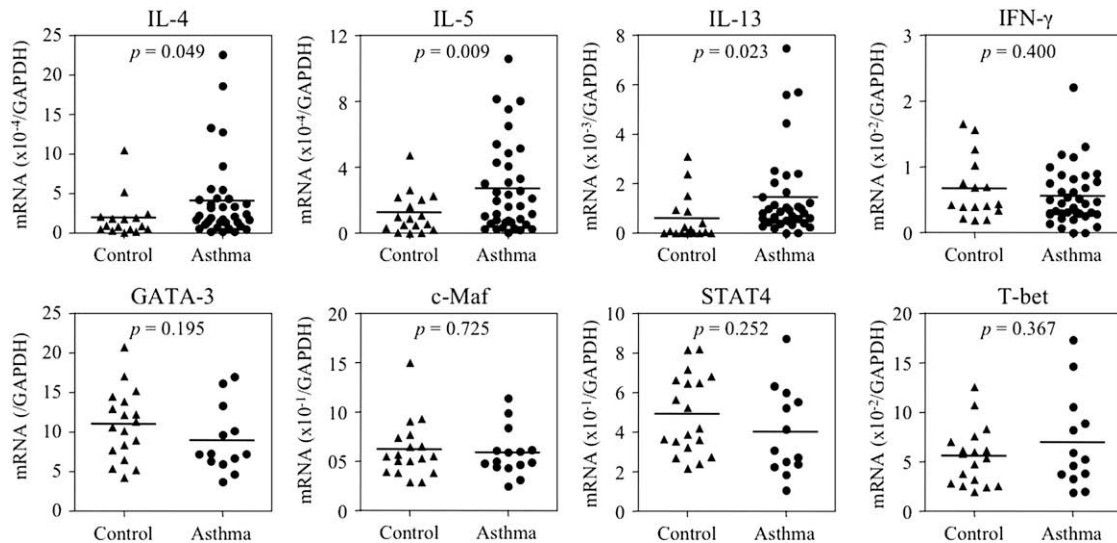


FIG 1. Expression of T_H1/T_H2 cytokines and transcription factors in $CD4^+$ T cells of asthmatic patients. mRNA expression in freshly isolated peripheral $CD4^+$ T cells of asthmatic patients and healthy donors was determined by using the quantitative real-time RT-PCR method. Each dataset (plot) and mean (bar) is expressed as mRNA abundance normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression ($n = 16-38$). P values were determined by using the unpaired t test with the Welch correction.

hardly observed in both peripheral and cord blood naive $CD4^+$ T cells and slightly induced on stimulation, whereas it was markedly upregulated by T_H2 differentiation. The expression of c-Maf was relatively stable. In contrast, STAT4 expression was clearly upregulated after T_H1 differentiation in both peripheral and cord blood $CD4^+$ T cells. In cord blood-derived cells, T-bet was also inducible and preferentially expressed in T_H1 cells. However, peripheral $CD4^+$ T cells displayed an interesting expression pattern of this transcription factor. Thus the expression of T-bet was not only equivalent in T_H1 and T_H2 cells but was also clearly detectable in naive $CD4^+$ T cells, even without stimulation (Fig 3, A).

It has been demonstrated that T-bet downregulated the transcriptional function of its opposing transcription factor, GATA-3.²⁴ Phosphorylation of a tyrosine residue in T-bet was required for the suppression of GATA-3.²⁴ Therefore phosphorylation, as well as coexpression with GATA-3, of T-bet in T_H2 cells derived from peripheral $CD4^+$ T cells in which both transcription factors were plentifully expressed was investigated. The expression of GATA-3 and T-bet in the cells was detectable also by means of intracellular staining (Fig 3, B). In addition, the expression levels of T-bet in GATA-3-low and GATA-3-high populations were equivalent, suggesting that T-bet was coexpressed with GATA-3 and its expression was not affected by GATA-3. Physical interaction between GATA-3 and T-bet was not detectable in these cells by means of immunoprecipitation with anti-T-bet or anti-GATA-3 antibodies in our experimental conditions (data not shown). However, T-bet was significantly phosphorylated in T_H1 and T_H2 cells derived from peripheral $CD4^+$ T cells (Fig 3, C). Interestingly, the phosphorylation level of T-bet was more potent in T_H2 cells than in T_H1 cells, which is in parallel with the expression pattern of GATA-3.

REA assay was performed throughout the *TBX21* gene to clarify the mechanisms of the hyperexpression of T-bet in peripheral $CD4^+$ T cells. Increasing amounts of a restriction

enzyme, *HaeIII*, degraded its recognition sequence in the distal promoter region (-1753) of peripheral and cord blood-derived T_H1/T_H2 -differentiated cells and peripheral naive $CD4^+$ cells but not that of cord blood naive cells (Fig 3, D). In accordance with the expression pattern of T-bet determined by means of Western blotting (Fig 3, A), the proximal promoter region (-151) was sensitive to the enzyme in peripheral blood cells, as well as in T_H1 cells from cord blood, but not in cord blood-derived naive and T_H2 cells. A similar degree of accessibility among peripheral and cord blood-derived naive/ T_H1 / T_H2 cells was observed in the first intron ($+1790$) of the *TBX21* gene (Fig 3, D). These findings suggest that the high accessibility of the proximal promoter region in the *TBX21* gene is one of the primary reasons for hyperexpression of T-bet in peripheral $CD4^+$ T cells.

Effect of T-bet on cytokine expression in human $CD4^+$ T cells

Subsequent experiments were performed to investigate the potential of T-bet, as well as other transcription factors, for T_H1 and T_H2 cytokine expression in human $CD4^+$ T cells. T-bet combined with internal ribosomal entry site sequence-Venus was introduced into cord blood naive $CD4^+$ T cells by means of a lentivirus infection system during differentiation toward T_H2 cells. Determined by the fluorescence of Venus, 30% to 45% of the resulting cells were recognized to be transduction positive, and the fluorescence levels were not significantly different between empty vector- and T-bet-introduced cells (Fig 4, A). In the population of transduction-positive cells, IL-4, but not IFN- γ , was clearly produced on stimulation, whereas less than 1% were of these cells were cytokine positive without stimulation (Fig 4, A). The IL-4-positive cells were slightly decreased in T-bet-transduced cells, and more distinctly, a large number of IFN- γ -producing cells were induced by T-bet (Fig 4, A). These

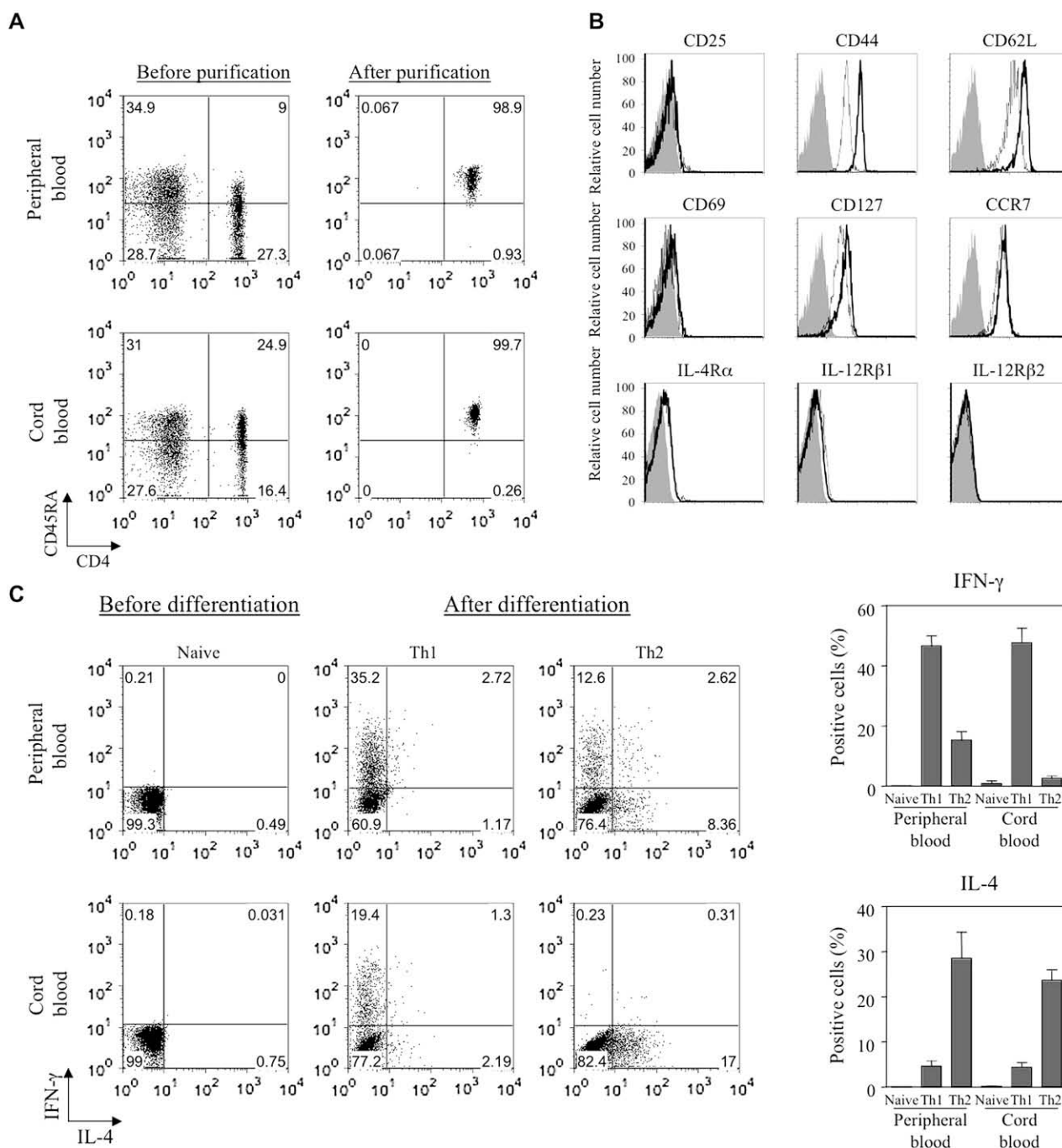


FIG 2. T_H1 and T_H2 cell differentiation from peripheral and cord blood naive T cells. CD45RA⁺CD4⁺ cells were purified from mononuclear cells in peripheral and cord blood of healthy subjects and stained for CD4 and CD45RA (**A**). Cell-surface markers on the peripheral (plain line) and cord (bold line) blood CD4⁺CD45RA⁺ cells were stained with specific or isotype control antibodies (gray area; **B**). After 7 to 10 days of stimulation culture under T_H1- and T_H2-skewing conditions, peripheral and cord blood CD4⁺ T cells were stimulated with 5 nmol/L phorbol 12-myristate 13-acetate plus 1 μ mol/L ionomycin for 6 hours and stained for intracellular IL-4 and IFN- γ (**C**). The representative plots and means $\pm$ SEMs of cytokine-positive cell percentages from 4 separate experiments are shown.

findings suggest that T-bet provides IFN- γ -producing activity for cord blood CD4⁺ T cells, even in T_H2-skewing conditions.

The effects of T-bet and other transcription factors on T_H1/T_H2 cytokine mRNA expression were examined. In this experiment peripheral naive CD4⁺ T cells were used because they expressed

both T_H1 and T_H2 cytokines after stimulation culture under neutral conditions. GATA-3, c-Maf, STAT4, and T-bet were introduced into the cells by using a lentivirus. Twenty percent to 30% of the resulting cells were recognized to be transduction positive, and populations with the same fluorescence level (M1; Fig 4, B)

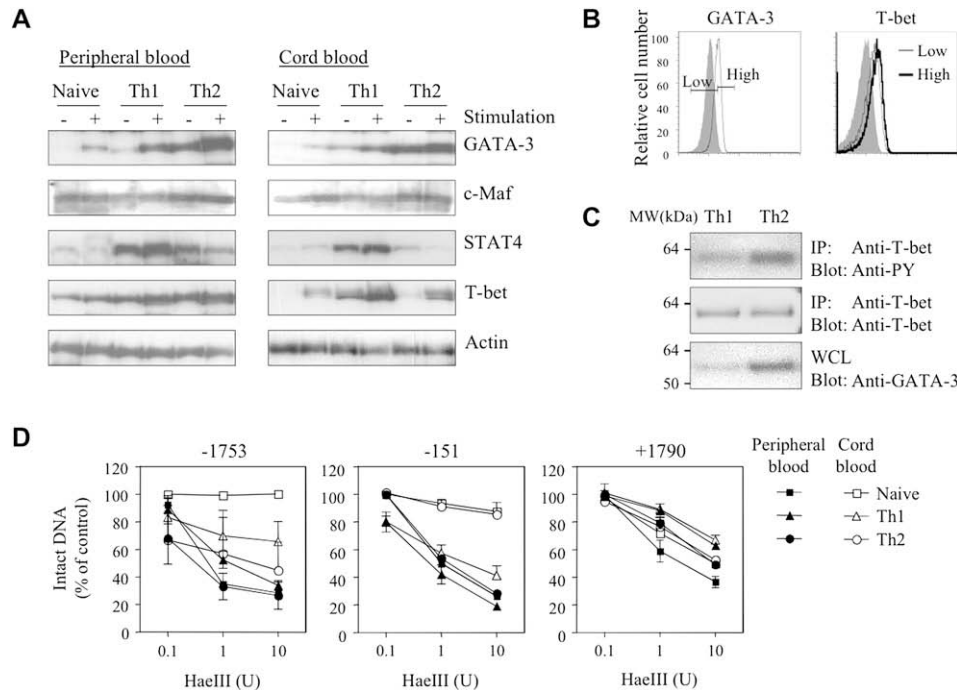


FIG 3. Expression of transcription factors in T cells. Purified naive $CD4^+$ T cells and differentiated T_H1 and T_H2 cells from peripheral and cord blood of healthy subjects were left unstimulated or stimulated with $0.1 \mu\text{g/mL}$ anti-CD3 plus $1 \mu\text{g/mL}$ anti-CD28 for 24 hours. Expression of transcription factors and actin in the resulting cells was analyzed by means of Western blotting. Protein samples from 10^6 cells of individual groups were loaded and blotted on the same membrane (**A**). GATA-3 and T-bet expression in T_H2 cells from peripheral $CD4^+$ T cells was examined by means of intracellular staining (**B**). Whole cell lysates (WCL) of T_H1 and T_H2 cells from peripheral blood $CD4^+$ T cells were immunoprecipitated with anti-T-bet antibody. The resulting immunoprecipitation (IP) samples or WCL were blotted with anti-phosphotyrosine, anti-T-bet, and anti-GATA-3 antibodies (**C**). The results shown are representative of 4 separate experiments. REA assay against *HaeIII* sites at the *TBX21* gene was performed on unstimulated naive and T_H1 - and T_H2 -differentiated peripheral and cord blood T cells (**D**). Data are expressed as means $\pm$ SEMs of percentage intact DNA compared with *HaeIII*-untreated control values ($n = 4-6$). MW, Molecular weight.

were purified by using a cell sorting system. On stimulation, the expression of both T_H1 and T_H2 cytokines was induced in these cells (Fig 4, C). IL-4, IL-5, and IFN- γ expression was upregulated by c-Maf, GATA-3, and T-bet, respectively. STAT4 failed to affect any cytokine examined. Not only marked augmentation of IFN- γ but also downregulation of IL-4 (by 81%) and IL-13 (by 34%) was achieved by T-bet. The specific expression of introduced GATA-3, c-Maf, STAT4, and T-bet was confirmed at the mRNA level (Fig 4, C).

Role of endogenously expressed T-bet in cytokine synthesis in peripheral $CD4^+$ T cells examined with RNAi technology

U6 promoter-driven shRNA against T-bet combined with EF promoter-driven Venus (shT-bet; Fig 5, A) was introduced into peripheral blood naive $CD4^+$ T cells by using a lentivirus during stimulation culture under neutral conditions. The expression of T-bet in highly transduced cells (M1; Fig 5, B) purified with a cell sorting system was examined by means of real-time RT-PCR and Western blotting. In comparison with nontargeted shRNA control, introduction of shT-bet reduced the expression of T-bet in the cells by approximately 80% at the level of both mRNA (Fig 5, C) and protein (Fig 5, D). As the protein content control,

equivalent expression of actin was observed in shT-bet- and control shRNA-introduced cells, whereas GATA-3 was slightly upregulated, if at all, by knockdown of T-bet (Fig 5, D). After stimulation culture, activated peripheral $CD4^+$ T cells produced IFN- γ , IL-4, and IL-13 in the culture supernatant (Fig 5, E). The production of IFN- γ was clearly suppressed, and on the contrary, IL-4 and IL-13 production was enhanced by the introduction of shT-bet (Fig 5, E). These findings suggest that endogenously expressed T-bet plays a crucial role in facilitating IFN- γ production in peripheral $CD4^+$ T cells.

Role of T-bet in T_H1/T_H2 cytokine gene transcription

In addition to the strong augmentation of IFN- γ , a suppressive role of T-bet in IL-4 and IL-13 was demonstrated in human $CD4^+$ T cells (Figs 4 and 5). Because T_H1 and T_H2 cytokines counteract each other, it is not clear whether T-bet-mediated downregulation of T_H2 cytokines was due to the suppression of T_H2 cytokine gene transcription or occurred as a secondary response after T_H1 polarization. The next experiment was therefore performed to elucidate the role of T-bet in IL-4 and IL-13 gene transcription. Jurkat Tag cells expressing both T_H1 and T_H2 cytokines on stimulation were used to separate this role from the overall effects on T_H1/T_H2 subset differentiation. pMACS-T-bet- and its empty

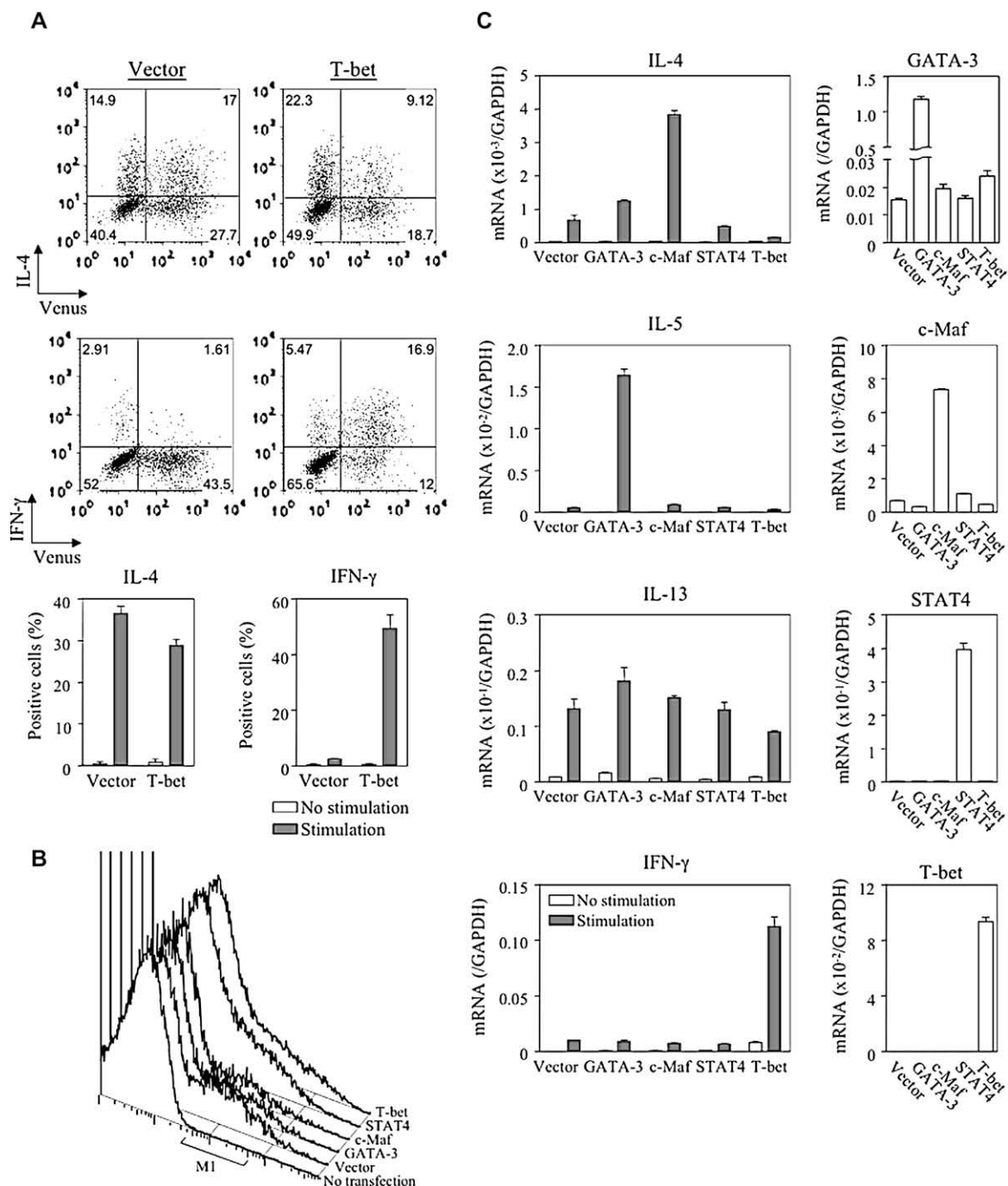


FIG 4. Effect of T-bet on cytokine synthesis in T cells. T-bet–internal ribosomal entry site sequence–Venus (*T-bet*) and empty internal ribosomal entry site sequence–Venus (*Vector*) expression cassettes were introduced into cord blood naive CD4⁺ T cells of healthy subjects by using a lentivirus infection system during 7 to 10 days of stimulation culture under T_H2 conditions. The resulting cells were stimulated with 5 nmol/L phorbol 12-myristate 13-acetate plus 1 μ mol/L ionomycin for 6 hours and stained for intracellular IL-4 and IFN- γ (**A**). The representative plots and means $\pm$ SEMs of cytokine-positive cell percentages in the Venus-positive population from 4 separate experiments are shown. Internal ribosomal entry site sequence–Venus–combined GATA-3, c-Maf, STAT4, and T-bet expression cassettes were introduced into peripheral naive CD4⁺ T cells during stimulation culture under neutral conditions. The resulting Venus-positive cells (M1 population; **B**) were purified and left unstimulated or stimulated with 5 nmol/L phorbol 12-myristate 13-acetate plus 1 μ mol/L ionomycin for 6 hours, and the expression of cytokine and transcription factor mRNA was measured by means of quantitative real-time RT-PCR (**C**). Data are expressed as means $\pm$ SEMs of mRNA abundance normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression (n = 4).

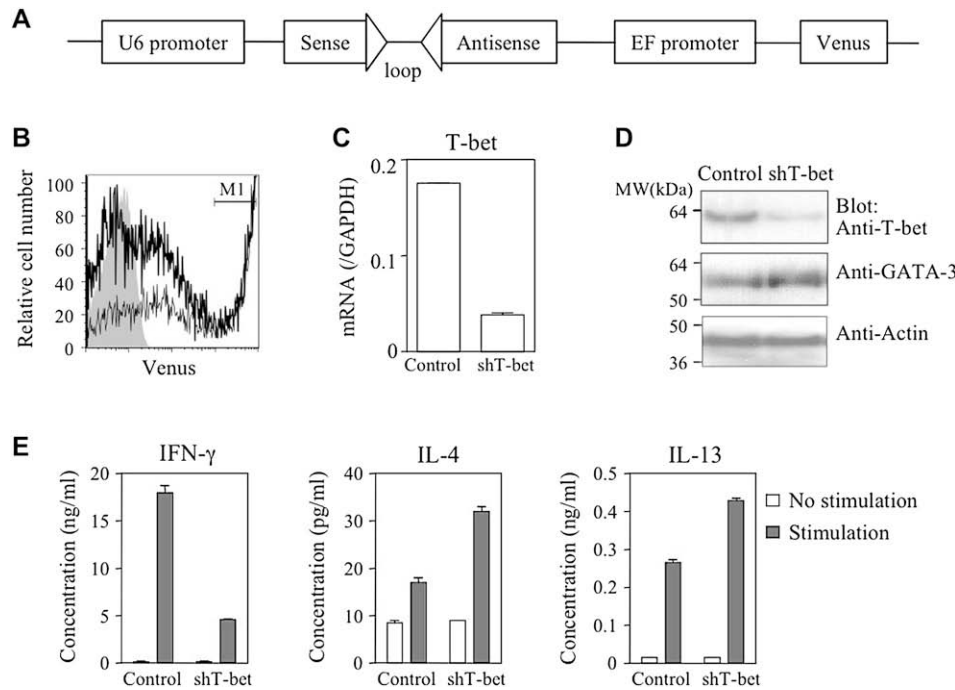


FIG 5. Knockdown effect of T-bet on human T-cell differentiation. A serial expression cassette containing U6 promoter-driven shRNA and EF promoter-driven Venus (**A**) was introduced into peripheral naive CD4⁺ T cells of healthy subjects with lentivirus during 7 to 10 days of stimulation culture under neutral conditions. The resulting Venus-positive population (M1 population; **B**) in T-bet (bold line) and control (plain line) shRNA-introduced cells was purified. The gray area indicates untransfected background. Expression of T-bet, GATA-3, or both in the purified cells was determined by means of quantitative real-time RT-PCR (**C**) and Western blotting (**D**). The purified cells were left unstimulated or stimulated with 0.1 μ g/mL anti-CD3 plus 1 μ g/mL anti-CD28 for 24 hours, and the concentrations of cytokines in the culture supernatant were measured by means of ELISA (**E**).

vector-transfected Jurkat Tag cells were purified by using a magnetic cell sorting system and stimulated in the presence of anti-IFN- γ antibody. As shown in Fig 6, A, T-bet enhanced inducible IFN- γ expression and suppressed the expression of IL-4 and IL-13. GATA-3, c-Maf, and STAT4 mRNA expression was not affected by overexpression of T-bet.

The effect of T-bet on T_H1/T_H2 cytokine gene transcription was further investigated by using a promoter reporter assay. Consistent with previous reports,^{21,25} the transcriptional activity of IFN- γ , IL4, and IL13 promoter was enhanced on stimulation. In agreement with the results of mRNA expression, the inducible promoter activity of IFN- γ was augmented, whereas that of IL-4 and IL-13 was inhibited by T-bet (Fig 6, B). These findings suggest that T-bet not only behaves as a strong transcription factor for IFN- γ but also downregulates T_H2 cytokines at the level of gene transcription in human T cells.

DISCUSSION

Accumulating evidence suggests that T_H2 cytokines are upregulated in asthmatic patients. These observations have been made not only in bronchial biopsy specimens but also in bronchoalveolar lavage fluid, peripheral blood, and CD4⁺ T cells.^{6,26-29} However, regardless of the T_H1/T_H2 paradigm, IFN- γ is not always suppressed in allergic asthma. Thus in some studies increased IFN- γ mRNA, protein, or both was detectable in bronchoalveolar lavage fluid, biopsy specimens, and peripheral blood.^{6,9} Our present study clearly demonstrated that IFN- γ

mRNA in CD4⁺ T cells of asthmatic patients was expressed equivalently to that in healthy subjects, even though T_H2 cytokines were upregulated, supporting the previous investigations.

Because it has been reported that cord blood T cells display lower IFN- γ -producing activity than peripheral T cells,¹³⁻¹⁵ we investigated the mechanisms of the distorted human T_H2 differentiation by comparing peripheral and cord blood CD4⁺ T cells. Although T_H1- and T_H2-polarized cells had clearly developed from cord blood naive CD4⁺ T cells and the T_H1 phenotype was also induced from peripheral cells, IFN- γ -producing cells had significantly developed from peripheral naive CD4⁺ T cells, even under T_H2-skewing conditions. Our present findings are consistent with those of previous reports¹³⁻¹⁵ and further suggest that the difference between peripheral and cord blood CD4⁺ T cells is already destined in their naive stage.

In relation to the distinct producing activity of T_H1 and T_H2 cytokines, a differential expression pattern in peripheral and cord blood CD4⁺ T cells was observed for T-bet. Thus T-bet was not detectable in unstimulated cord blood naive CD4⁺ T cells and was preferentially induced after T_H1 differentiation. In contrast, the expression of T-bet was not only equivalent in T_H1 and T_H2 cells but was also detectable even in resting naive CD4⁺ T cells derived from peripheral blood. It has been reported that several transcription factors are differentially expressed in peripheral and cord blood T cells.^{12,16,17} Partly in agreement with and partly in contradiction to our present findings, Yu et al¹² demonstrated that T-bet and GATA-3 mRNA expression was enhanced and diminished, respectively, in mononuclear cells of peripheral blood

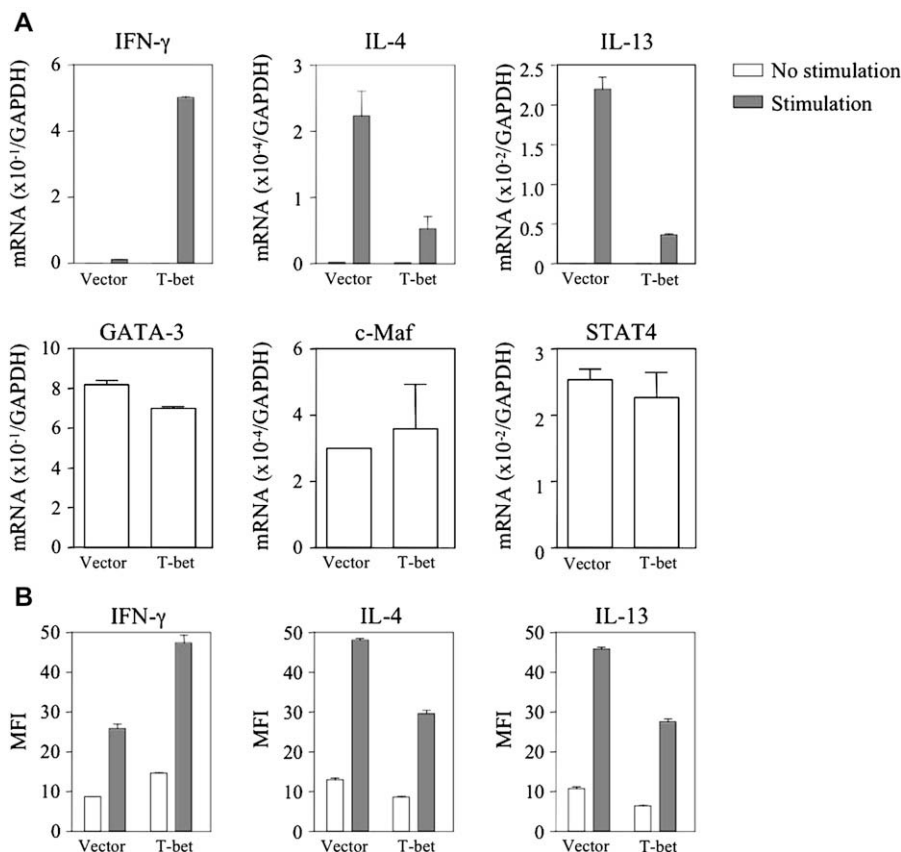


FIG 6. Effect of T-bet on cytokine mRNA expression and promoter activity in human T cells. **A**, Jurkat Tag cells were transfected with pMACS-T-bet or empty vector (10 μ g each). After 48 hours, H-2k-positive cells purified with a magnetic cell sorting system were left unstimulated or stimulated with phorbol 12-myristate 13-acetate (5 nmol/L) plus ionomycin (1 μ mol/L) in the presence of 40 μ g/mL anti-IFN- γ for 6 hours, and the expression of cytokine and transcription factor mRNA was determined by using the quantitative real-time RT-PCR method. Data are expressed as means $\pm$ SEMs of mRNA abundance normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression ($n = 4$). **B**, Cells were transfected with pEF-T-bet or empty vector in the presence of IFN- γ -EGFP, IL-4-EGFP, or IL-13-EGFP (10 μ g each). At 24 hours after transfection, cells were stimulated in the presence of 40 μ g/mL anti-IFN- γ for 16 hours, and the promoter activity was detected as the fluorescence of synthetic EGFP measured by means of flow cytometry ($n = 4$).

but not cord blood on stimulation with varicella zoster virus. Our study cannot be directly compared with their report because they examined mRNA levels and only discussed the stimulation/no stimulation ratio in the whole mononuclear cell population. However, we have observed that the expression levels of T_H1- and T_H2-specific transcription factors as proteins were not correlated with those as mRNA (see Fig E1 in this article's Online Repository at www.jacionline.org). Furthermore, mRNA expression levels of transcription factors were not consistent with the hyperexpression of T_H2 cytokines in asthmatic patients (Fig 1). Therefore the present findings demonstrating a differential T-bet expression pattern in purified peripheral and cord blood CD4⁺ T-cell populations at the protein level seem to be more convincing and, more importantly, consistent with the results of the T_H1/T_H2 differentiation experiment.

The importance of T-bet to T_H1 differentiation has been reported. T cells derived from T-bet-deficient mice showed defective T_H1 differentiation,³⁰ and accordingly, these mice spontaneously manifested T_H2-related disorders.³¹ Ectopically expressed T-bet induced IFN- γ synthesis in T_H2-polarized cells of murine and human origin.^{32,33} The expression of T-bet in T cells is

correlated with polarization of the murine T_H1 phenotype,³² although the correlation has been poorly confirmed in human T cells. Consistent with these previous investigations, our present study clearly demonstrated that IFN- γ -producing activity in human CD4⁺ T cells is generally correlated with the resting expression pattern of T-bet protein.

We also demonstrated that, like murine T cells, ectopically expressed T-bet induced IFN- γ synthesis in human CD4⁺ T cells, even after stimulation culture under T_H2-polarizing conditions. Furthermore, the functional role of endogenously expressed T-bet in IFN- γ synthesis was clearly demonstrated by using RNAi technology. Consistently, Lametschwandner et al³³ reported that ectopic expression of T-bet in a human T_H2 cell line derived from the skin of patients with atopic dermatitis induced IFN- γ -producing activity. Therefore it is suggested that T-bet plays a crucial role in the expression of IFN- γ in human CD4⁺ T cells, even under a T_H2-skewing state, such as allergic asthma.

Nevertheless, peripheral naive CD4⁺ T cells did not produce IFN- γ , regardless of significant expression of T-bet. In addition to the requirement of other transcription factors, such as nuclear factor of activated T cells, nuclear factor κ B, and activator protein

1, IFN- γ gene transcription is strongly affected by T-cell receptor signaling–triggered epigenetic modification of its gene locus.^{34,35} Therefore T-bet might hardly associate with and activate the IFN- γ promoter in peripheral naive CD4⁺ T cells.

The expression of T-bet also seems to be regulated at the level of transcription with epigenetic modification of its gene locus. Indeed, T-bet was strongly inducible on stimulation and significantly expressed also in naive and T_H2 cells at 24 hours (Fig 3, A). However, the *de novo* synthesized T-bet did not contribute to the expression of IFN- γ (Fig 2, C). The correct reason for the discrepancy is not clear, though it is probably due to the difference in time course because cytokine-producing activity was determined in an earlier time point (6 hours). In addition, our present findings suggest that the protein expression levels of transcription factors at the start of stimulation are crucial for inducible T_H1/T_H2 cytokine synthesis.

We and Lametschwandner et al³³ demonstrated apparently contradictory findings. Thus in addition to the strong promoting activity on IFN- γ , downregulation of IL-4 and IL-13 by T-bet was observed in our present study, whereas Lametschwandner et al showed that IL-4 synthesis was not affected by ectopic expression of T-bet in human T_H2 cells. The reason for the contradiction is unclear, although our present study further demonstrated that T-bet–mediated downregulation of IL-4 and IL-13 was achieved at the mRNA transcription level. In agreement with our findings, IL-4 and IL-13 production by CD4⁺ T cells was upregulated in T-bet–deficient mice.³⁶ Even though T_H1 and T_H2 cytokines counteract each other, downregulation of IL-4 and IL-13 by T-bet was not likely to be achieved, at least in part, through the enhancement of IFN- γ expression because Jurkat Tag cells failed to produce a detectable amount of IFN- γ , as well as IL-4, IL-5, and IL-13, in the culture supernatant (<20 pg by 10⁶ cells).

Nevertheless, Szabo et al³⁰ showed that T-bet failed to affect the promoter activity of murine IL-4, even though IFN- γ promoter activity was augmented. They used a reporter construct containing –760 to +68 of the murine *IL4* promoter and murine EL-4 cells, whereas we used a human T leukemia cell line. Because the homology of the approximately 1-kb 5′-flanking regions of the human and mouse *IL4* genes is approximately 60%, the effect of T-bet on the *IL4* promoter might vary among species. Because T-bet–binding activity of the human and murine *IL4* and *IL13* gene promoters, including the T-box–like region, has not been evaluated, further study will be needed to identify the cis-regulatory elements responsible for T-bet–mediated downregulation of IL-4 and IL-13 gene transcription.

Recently, the counteractive effects of T-bet on T_H2-specific transcription factors have been investigated. GATA-3 expression in murine CD4⁺ T cells was upregulated in T-bet^{–/–} mice and, on the contrary, suppressed by retroviral introduction of T-bet.³⁷ Our present study revealed new insights into the role of T-bet in GATA-3 expression in human CD4⁺ T cells. Thus the expression levels of T-bet in GATA-3–low and GATA-3–high populations in T_H2 cells derived from peripheral blood CD4⁺ T cells are equivalent. In addition, GATA-3 mRNA expression was minimally affected by ectopic expression of T-bet in peripheral T cells and Jurkat Tag cells (Figs 4, C, and 6, A), suggesting that T-bet does not directly affect GATA-3 expression in human T cells. Because the expression of GATA-3 was slightly enhanced by knockdown of T-bet (Fig 5, D), this is probably due to a secondary effect caused by the relative polarization to the T_H2 phenotype.

The phosphorylation of T-bet was more potent in T_H2 cells than in T_H1 cells, which is in parallel with the expression of GATA-3 (Fig 3, C). It has been reported that IL-2-inducible T-cell kinase (ITK) is necessary for GATA-3 association and phosphorylation of T-bet.²⁴ Therefore the possibility that GATA-3 is required for the interaction with ITK and the resulting phosphorylation of T-bet is also suggested. Because the physiologic meaning of hyperphosphorylation of T-bet in T_H2 cells is unclear in our study, Hwang et al²⁴ showed that phosphorylated T-bet interferes with the binding of GATA-3 to its target DNA. Therefore a negative contribution of T-bet, expressed and phosphorylated in T_H2 cells derived from peripheral blood, to GATA-3–mediated T_H2 cytokine synthesis is suggested.

In conclusion, T-bet expressed in human peripheral CD4⁺ T cells plays a crucial role in their IFN- γ productivity, even in T_H2-skewing conditions, through not only strong induction of IFN- γ but also downregulation of IL-4 and IL-13 at the transcription level. Hyperexpression of this transcription factor in naive CD4⁺ T cells because of the high accessibility to the proximal *TBX21* promoter region might be one of the primary reasons for the lack of downregulation of IFN- γ , regardless of upregulation of T_H2 cytokines, in T_H2 diseases, including allergic asthma.

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Key message

- The T_H1/T_H2 paradigm established principally by murine studies is not always fit for the differentiation of human T cells.

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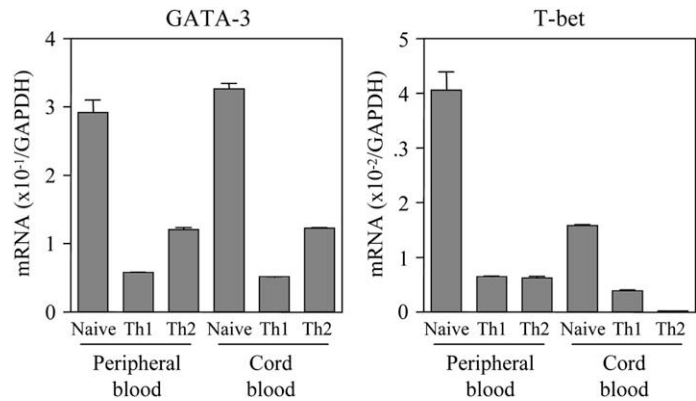


FIG E1. mRNA expression of GATA-3 and T-bet in peripheral and cord blood CD4⁺ T cells. Purified naive CD4⁺ T cells and differentiated T_H1 and T_H2 cells from peripheral and cord blood of healthy subjects was determined by means of quantitative real-time RT-PCR method. Data are expressed as means $\pm$ SEMs of mRNA abundance normalized with glyceraldehyde-3-phosphate dehydrogenase (*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- γ	GAAATATTTTAATGCAGGTCATTCA
GATA-3	GACCCACCACGGGAGCCAGGTGTGC
c-Maf	GTTTTTCATAACTGAGCCCACTCGC
STAT4	GTAAAGGAAATGAGGGCTGTCACAT
T-bet	CAAGCAGGGACGGCGGATGTTCCCA

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

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