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

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This work was supported in part by the Japan Allergy Foundation (to K.O.).

25 **Abstract**

26

27 **Background:** Regardless of Th1/Th2 theory, $CD4^+$ T cells of patients with allergic asthma, a
28 typical Th2 disease, and those of healthy subjects expressed equivalent levels of IFN-γ, even
29 though Th2 cytokines were significantly up-regulated in asthmatic patients.
30 **Objective:** The mechanisms underlying distorted Th2 cell polarization in human T cells were
31 elucidated.
32 **Methods:** Cytokine-producing activity and the expression of Th1/Th2-specific transcription
33 factors in naïve and/or Th1/Th2 $CD4^+$ T cells derived from human peripheral and cord blood
34 were comparatively analyzed. The mechanisms of the differential expression of T-bet in the cells
35 were assessed by determining the chromatin accessibility at the T-bet gene. The functional roles
36 of T-bet and other transcription factors in human Th1/Th2 differentiation were further
37 investigated.
38 **Results:** Th2 cells derived from naïve $CD4^+$ T cells in peripheral blood but not in cord blood
39 produced IFN-γ. T-bet was expressed in peripheral but not cord blood resting naïve T cells.
40 Consistently, the accessibility at the proximal T-bet gene promoter in peripheral naïve T cells was
41 higher than that in cord blood naïve T cells. IFN-γ-producing activity was induced in
42 Th2-differentiated cord blood T cells by ectopic expression of T-bet. In addition, a reduction of
43 T-bet in peripheral T cells suppressed IFN-γ production. T-bet not only up-regulated IFN-γ, but
44 also down-regulated IL-4 and IL-13 gene transcription, independently of the modification of
45 Th1/Th2 balance.
46 **Conclusion:** The expression of T-bet at a naive stage is crucial for the development of
47 IFN-γ-producing T cells in human peripheral blood even in Th2-related diseases.
48

Key Messages

The Th1/Th2 paradigm established principally by murine studies is not always fit for the differentiation of human T cells.

Capsule Summary

Hyper-expression of T-bet in human peripheral $CD4^+$ T cells, due to high accessibility to the proximal T-bet promoter region, may be one of the primary reasons for the lack of down-regulation of IFN-γ, regardless of up-regulation of Th2 cytokines, in Th2-diseases.

Key Words

Allergy, Bronchial Asthma, Cord Blood, IFN-γ, IL-4, Human T cells, T-bet, Th1/Th2.

Abbreviations

IP; immunoprecipitation, IRES; internal ribosomal entry site sequence, REA; restriction enzyme accessibility, WCL; whole cell lylsates.

Introduction

$CD4^+$ T cells play a central role in immune responses. Among currently recognized at least four distinct $CD4^+$ T cell subsets, Th1, Th2, Th17 and regulatory T cells, Th1 and Th2 subpopulations were identified about two decades ago and have been extensively investigated so far [1, 2]. Th1 cells specifically produce IFN-γ, which controls cellular immunity, while Th2 cells preferentially secrete IL-4, IL-5 and IL-13, which regulates humoral immunity [2, 3]. Impairment of Th1/Th2 balance has been suggested to cause a variety of diseases. For example, acceleration of Th1 response is recognized in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, whereas excessive Th2 responses are observed in allergic diseases including allergic asthma and atopic dermatitis [2, 3]. This Th1/Th2 paradigm has led to the concept that immunotherapy converting Th2 into Th1 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 Th1/Th2 dichotomy [5]. For example, bronchial biopsy specimens from asthmatic patients are characterized by $CD4^+$ T cells displaying Th2 cytokines [6], suggesting that allergic asthma is considered as a representative Th2 disease. Nevertheless, although there may be a reduced presence of Th1 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 al. showed that elevated serum IFN-γ correlated with airway hyper-responsiveness in asthmatic patients [9]. 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 Th2 cells.

Although the reason for the inconsistency of the Th1/Th2 paradigm in human $CD4^+$ T cells has been poorly elucidated, neonates are known to display immature immunity to infections partly due to poor Th1 responses [11, 12]. It has been demonstrated that 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 Th1- and Th2-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 helpful for understanding the mechanisms of the distorted Th2 differentiation in human Th2-related diseases.

In accordance with previous findings, we here investigated the increased expression of Th2 cytokines, but not down-regulation of IFN-γ in $CD4^+$ T cells of patients with allergic asthma. The significant IFN-γ-producing property even under Th2 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 Th1/Th2-specific transcription factors by western blot and restriction enzyme accessibility (REA) assay. A crucial role of T-bet in Th1/Th2 differentiation was demonstrated in overexpression and knockdown studies by employing 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 Sagamihara National Hospital (Kanagawa, Japan). Thirty-seven adult patients referred to the outpatient clinic in these hospitals (male = 17, female = 20, age: mean ± 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 FEV1.0 after β_2-agonist inhalation. Total serum IgE level was 652 ± 728 IU/ml. Treatment with β_2-agonists, anticholinergics and theophylline was stopped at least 12 h before blood sampling. Patients receiving corticosteroids were excluded from the study. Human umbilical cord blood was collected at the time of elective Caesarean sections or normal spontaneous delivery of non-allergic healthy mothers. Written informed consent was obtained from all subjects, including 18 healthy volunteers (male = 10, female = 8, age: 62.4 ± 12.3 years), prior to investigations.

Plasmid Constructs

The PCR fragment of cDNA encoding full-length human T-bet (a.a. 1-533), GATA-3 (1-443), c-Maf (1-373) and STAT4 (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 (IRES) 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 Biotech GmbH, Bergisch Gladbach, Germany) was replaced with the CMV promoter plus/minus T-bet cDNA-coding sequence (pMACS-T-bet). T-bet was also subcloned into the

pEF6/His vector (Invitrogen, Carlsbad, CA) (pEF-Tbet). To construct a 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'-CGCTTCCAACACGCATATCTT-3') and that in its non-target shRNA control vector (Sigma-Aldrich, St. Louis, MO) were replaced by human elongation factor 1α promoter-driven Venus expression cassette [19, 20]. The human IL-4 promoter (-1105 to +4) and IL-13 promoter (-1077 to +49) were subcloned into the pEGFP-1 vector (BD Bioscience, San Jose, CA) (IL-4-EGFP and IL-13-EGFP). The human IFN-γ promoter-reporter vector (IFN-γ-EGFP) was described previously [21]. The correct sequences of all constructs were verified by 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 three plasmids: packaging construct (pCAG-HIVgp), VSV-G and Rev-expressing construct (pCMV-VSV-G-RSV-Rev) and lentivirus vectors described above. The resulting culture supernatant was concentrated by ultracentrifugation, and the virus pellet was re-suspended 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 positive selection using CD4 microbeads with a magnetic cell sorting system (Miltenyi). The purity of resulting cells was >95% as determined by flow cytometry. $CD4^+CD45RO^-$ naïve T cells were also purified by a combination of negative and positive selection using CD45RO and CD4

158 microbeads, respectively. The resulting cells were analyzed for the expression of cell surface
159 molecules by staining with FITC- or PE-conjugated anti-CD25, -CD44, -CD62L, -CD69, -CD127
160 (eBioscience, San Diego, CA), -CCR7, -IL-4 receptor (R) α, IL-12Rβ1 (R & D Systems,
161 Minneapolis, MN) and -IL-12Rβ2 (BD Bioscience) Abs. Naïve T cells were cultured in AIM-V
162 medium (Invitrogen) in the presence of 0.01 μg/ml anti-CD3 Ab (OKT3; Ortho Biotech,
163 Bridgewater, NJ), 1 μg/ml anti-CD28 Ab (BD Bioscience) and 10 U/ml recombinant IL-2
164 (Shionogi, Osaka, Japan). For Th1 phenotype development, 100 ng/ml recombinant IL-12
165 (Peprotech, Rocky Hill, NJ) and 40 μg/ml anti-IL-4 Ab (eBioscience) were added at the start of
166 culture, and for Th2 development, 400 ng/ml recombinant IL-4 (Peprotech) and 40 μg/ml anti
167 IL-12 and anti-IFN-γ Abs (eBioscience) were used. In neutralizing conditions, no other
168 recombinant cytokine or anti-cytokine Ab than IL-2 was added. For protein overexpression and
169 knockdown study, cells were infected with viral stock solutions at a multiplicity of infection
170 (MOI) of 50 to 200 at 24 h after the start of culture. After seven to ten days of stimulation, cells
171 were harvested, purified by centrifugation over Ficoll-Paque and then used as Th1/Th2-polarized
172 or neutrally developed cells. In some experiments, lentivirus infection-positive cells were purified
173 using a FACSArea cell-sorting system (BD Bioscience).
174 Simian virus 40 T antigen-transfected human leukemic Jurkat T (Jurkat Tag) cells were
175 transfected with various amounts of plasmid DNAs, by electroporation as described previously [22].
176 In some experiments, transfection-positive cells were purified by positive selection using H-2^k
177 microbeads with a magnetic cell sorting system. The purity of resulting cells was >95% as
178 determined by flow cytometry with anti-H-2^k-FITC Ab.
179
180 **Immunoprecipitation and immunoblotting**
181 Whole cell lysates (WCL) prepared from 10^7 cells as described previously [22] were
182 incubated with 5 μg of anti-T-bet (4B10) for 2 h, followed by the addition of 40 μg protein

G-Sepharose beads for an additional 2 h at 4°C. The beads were then washed four times, boiled in 20 µl of Laemmli buffer and subjected to SDS-PAGE. Immunoblotting against endogenous protein in WCL and/or immnoprecipitation (IP) samples was performed 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, CA) and anti-phosphotyrosine (PY-20) (BD Bioscience) Abs as described [21].

Intracellular Cytokine Staining

After stimulation culture, $CD4^+$ T cells were restimulated with 5 nM PMA + 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 phosphate-buffered saline containing 3% BSA, cells were stained with anti-IL-4 (MP4-25D2)-FITC or -PE (eBioscience), anti-IFN-γ (4S.B3)-PE (BD Bioscience), anti-GATA-3-PE (BD Bioscience) and anti-T-bet-Alexa647 (eBioscience) Abs. Flow cytometric analysis was performed on a FACScalibur with CellQuest software (BD Bioscience).

Messenger RNA expression

Total RNA was extracted from freshly isolated $CD4^+$ T cells or cells stimulated with or without 2.5 µg/ml anti-CD3 Ab + 1 µg/ml anti-CD28 Ab in the presence of 5 µg/ml anti-mouse IgG crosslinking Ab or 5 nM PMA + ionomycin for 6 h at 37°C. After reverse transcription using 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 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, CA) as described previously [21].

Reporter Assay and ELISA

After 16 h of stimulation, the promoter-enhancer activity was assessed as fluorescence of synthetic EGFP detected by 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 ELISA using READY-SET-GO ELISA sets (eBioscience) according to the manufacturer's instructions.

Restriction Endonuclease Accessibility (REA) Assay

REA was performed according to the method of Guo et al. [23] with modifications. Briefly, 1 x 10^6 cells were washed twice in cold PBS and resuspended in lysis buffer (60 mM KCL, 15 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.5], 300 mM sucrose, and 0.625% NP-40) with freshly supplied protease inhibitors. After cells were lysed on ice for 10 min, the nuclei were pelleted at 2000 rpm for 10 min at 4°C. Nuclei were then treated with varying amounts of HaeIII digestion buffer (50 mM NaCl, 10 mM Tris-HCl [pH7.9], 10 mM $MgCl_2$ and 1 mM dithiothreitol) at 37°C for 24 h. The reactions were stopped by addition of EDTA to a final concentration of 10 mM. 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 h. 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 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 T-bet gene were described in Table E2 in this article's Online Repository. The results were expressed as the relative amount of intact DNA in comparison with non-enzyme-treated control.

Results

$CD4^+$ T cells are distortedly differentiated into Th2 cells in allergic asthma

Regardless of the Th1/Th2 theory, it has not been clearly evaluated whether Th1 cytokines are down-regulated in human Th2-related diseases. Therefore, the expression of Th1/Th2 cytokines and transcription factors in $CD4^+$ T cells of patients with allergic asthma, a typical Th2 disease, was first investigated. To exclude the difference in the antigen specificity among individual patients, mRNA expression levels in freshly isolated $CD4^+$ T cells from peripheral blood were measured by quantitative real-time RT-PCR. Consistent with previous reports [3, 4], IL-4, IL-5 and IL-13 expression 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 Th1-specific transcription factors, STAT4 and T-bet, and Th2 transcription factors, GATA-3 and c-Maf. These findings suggest that human $CD4^+$ T cells are not differentiated into typical Th2 cells even in Th2-related diseases.

Human peripheral naïve $CD4^+$ T cells-derived Th2 cells produce IFN-γ

To clarify the mechanisms underlying the lack of down-regulation of IFN-γ in allergic asthma, the potential for Th1/Th2 differentiation of peripheral naïve $CD4^+$ T cells was next investigated. In this experiment, naïve $CD4^+$ T cells prepared from cord blood were also employed, since it has been suggested that cord blood T cells display poor IFN-γ-producing activity [13-15]. As shown in Fig. 2A, peripheral blood and cord blood mononuclear cells contained ~9% and ~25% $CD45RA^+$ naïve $CD4^+$ T cells, respectively. From both mononuclear cells, $CD4^+CD45RA^+$ T cells were successfully enriched (~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. 2B), even though the expression levels of the positive molecules differed in individual subjects (data not shown), suggesting that these cells are recognized as typical naïve T cells. IL-4Rα and IL-12Rβ1 but not IL-12Rβ2 were similarly expressed on both cells. After stimulation culture under Th1 and Th2 conditions, respective phenotypes were clearly induced from cord blood naïve $CD4^+$ T cells (Fig. 2C). Th1 differentiation was also successfully induced, though peculiar Th2 differentiation occurred in peripheral blood naïve $CD4^+$ T cells. Thus, IFN-γ-producing cells were significantly developed even under Th2-skewing conditions (Fig. 2C).

Peripheral naïve $CD4^+$ T cells significantly express T-bet due to high accessibility of the proximal promoter region in the T-bet gene

In order to elucidate the reason for the distorted Th2 differentiation in human peripheral $CD4^+$ T cells, the expression of Th1- and Th2-specific transcription factors in peripheral and cord blood naïve/Th1/Th2 cells was comparatively analyzed by western blot. As shown in Fig. 3A, GATA-3 expression was hardly observed in both peripheral and cord blood naive $CD4^+$ T cells and slightly induced upon stimulation, whereas it was markedly up-regulated by Th2 differentiation. The expression of c-Maf was relatively stable. In contrast, STAT4 expression was clearly up-regulated after Th1 differentiation in both peripheral and cord blood $CD4^+$ T cells. In cord blood-derived cells, T-bet was also inducible and preferentially expressed in Th1 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 Th1 and Th2 cells, but was also clearly detectable in naive $CD4^+$ T cells even without stimulation (Fig. 3A).

It has been demonstrated that T-bet down-regulated 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 co-expression

283 with GATA-3 of T-bet in Th2 cells derived from peripheral $CD4^+$ T cells in which both
284 transcription factors were plentifully expressed was investigated. The expression of GATA-3 and
285 T-bet in the cells was detectable also by intracellular staining (Fig. 3B). In addition, the
286 expression levels of T-bet in GATA-3-low and -high populations were equivalent, suggesting that
287 T-bet was co-expressed with GATA-3 and its expression was not affected by GATA-3. Physical
288 interaction between GATA-3 and T-bet was not detectable in these cells by IP using anti-T-bet or
289 anti-GATA-3 Ab in our experimental conditions (data not shown). However, T-bet was
290 significantly posphorylated in Th1 and Th2 cells derived from peripheral $CD4^+$ T cells (Fig. 3C).
291 Interestingly, the phosphorylation level of T-bet was more potent in Th2 cells than Th1 cells, in
292 parallel with the expression pattern of GATA-3.

293 To clarify the mechanisms of the hyper-expression of T-bet in peripheral $CD4^+$ T cells,
294 REA assay was performed throughout the T-bet gene. Increasing amounts of a restriction enzyme,
295 HaeIII, degraded its recognition sequence in the distal promoter region (-1753) of peripheral and
296 cord blood-derived Th1/Th2-differentiated cells and peripheral naïve $CD4^+$ cells, but not that of
297 cord blood naïve cells (Fig. 3D). In accordance with the expression pattern of T-bet determined
298 by western blot (Fig. 3A), the proximal promoter region (-151) was sensitive to the enzyme in
299 peripheral blood cells as well as in Th1 cells from cord blood, but not in cord blood-derived naïve
300 and Th2 cells. A similar degree of accessibility among peripheral and cord blood-derived
301 naïve/Th1/Th2 cells was observed in the first intron (+1790) of the T-bet gene (Fig. 3D). These
302 findings suggest that the high accessibility of the proximal promoter region in the T-bet gene is
303 one of the primary reasons for hyper-expression of T-bet in peripheral $CD4^+$ T cells.
304
305 **Effect of T-bet on cytokine expression in human $CD4^+$ T cells**
306 Subsequent experiments were performed to investigate the potential of T-bet as well as
307 other transcription factors for Th1 and Th2 cytokine expression in human $CD4^+$ T cells. The
308 T-bet combined with IRES-Venus was introduced into cord blood naïve $CD4^+$ T cells by a

lentivirus infection system during differentiation toward Th2 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. 4A). In the population of transduction-positive cells, IL-4 but not IFN-γ was clearly produced upon stimulation, whereas <1% were cytokine positive without stimulation (Fig 4A). 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. 4A). These findings suggest that T-bet provides IFN-γ-producing activity for cord blood $CD4^+$ T cells even in Th2-skewing conditions.

The effects of T-bet and other transcription factors on Th1/Th2 cytokine mRNA expression were examined. In this experiment, peripheral naïve $CD4^+$ T cells were employed since they expressed both Th1 and Th2 cytokines after stimulation culture under neutral conditions. GATA-3, c-Maf, STAT4 and T-bet were introduced into the cells using a lentivirus. Twenty to 30% of the resulting cells were recognized to be transduction positive, and populations with the same fluorescence level (M1; Fig. 4B) were purified by a cell sorting system. Upon stimulation, the expression of both Th1 and Th2 cytokines was induced in these cells (Fig. 4C). IL-4, IL-5 and IFN-γ expression was up-regulated 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 down-regulation 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. 4C).

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

U6 promoter-driven shRNA against the T-bet combined with EF promoter-driven Venus (shT-bet; Fig. 5A) was introduced into peripheral blood naïve $CD4^+$ T cells using a lentivirus during stimulation culture under neutral conditions. The expression of T-bet in highly transduced cells (M1; Fig. 5B), purified by a cell sorting system, was examined by real-time RT-PCR and western blot. In comparison with non-targeted shRNA control, introduction of shT-bet reduced the expression of T-bet in the cells by ~80% at the level of both mRNA (Fig. 5C) and protein (Fig. 5D). As the protein content control, equivalent expression of actin was observed in shT-bet- and control shRNA-introduced cells, while GATA-3 was slightly up-regulated, if any, by knockdown of T-bet (Fig. 5D). After stimulation culture, activated peripheral $CD4^+$ T cells produced IFN-γ, IL-4 and IL-13 in the culture supernatant (Fig. 5E). 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. 5E). 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 Th1/Th2 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). As Th1 and Th2 cytokines counteract each other, it is not clear whether T-bet-mediated down-regulation of Th2 cytokines was due to the suppression of Th2 cytokine gene transcription, or occurred as a secondary response following Th1 polarization. The next experiment was therefore performed to elucidate the role of T-bet in IL-4 and IL-13 gene transcription. To separate this role from the overall effects on Th1/Th2 subset differentiation, Jurkat Tag cells expressing both Th1 and Th2 cytokines upon stimulation were employed. pMACS-T-bet- and its empty vector-transfected Jurkat Tag cells were purified by a magnetic cell sorting system and stimulated in the presence of anti-IFN-γ Ab. As shown in Fig. 6A, T-bet enhanced inducible IFN-γ expression, and on the contrary,

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 Th1/Th2 cytokine gene transcription was further investigated by employing a promoter reporter assay. Consistent with previous reports [21, 25], the transcriptional activity of IFN-γ, IL-4 and IL-13 promoter was enhanced upon 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. 6B). These findings suggest that T-bet not only behaves as a strong transcription factor for IFN-γ, but also down-regulates Th2 cytokines at the level of gene transcription in human T cells.

Discussion

Accumulating evidence suggests that Th2 cytokines are up-regulated 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 Th1/Th2 paradigm, IFN-γ is not always suppressed in allergic asthma. Thus, in some studies, increased IFN-γ mRNA and/or protein 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 Th2 cytokines were up-regulated, supporting the previous investigations.

As 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 Th2 differentiation by comparing peripheral and cord blood $CD4^+$ T cells. Although Th1- and Th2-polarized cells had clearly developed from cord blood naïve $CD4^+$ T cells, and the Th1 phenotype was also induced from peripheral cells, IFN-γ-producing cells had significantly developed from peripheral naïve $CD4^+$ T cells even under Th2-skewing conditions. Our present findings are consistent with previous reports [13-15], and further suggest that the difference between peripheral and cord blood $CD4^+$ T cells is already destined in their naïve stage.

In relation to the distinct producing activity of Th1 and Th2 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 naïve $CD4^+$ T cells, and was preferentially induced following Th1 differentiation. In contrast, the expression of T-bet was not only equivalent in Th1 and Th2 cells, but was also detectable even in resting naïve $CD4^+$ 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

393 our present findings, Yu et al. demonstrated that T-bet and GATA-3 mRNA expression was
394 enhanced and diminished, respectively, in mononuclear cells of peripheral blood but not cord
395 blood upon stimulation with varicella zoster virus [12]. Our study can not be directly compared with
396 their report, since they examined mRNA levels and only discussed the stimulation/no stimulation
397 ratio in the whole mononuclear cell population. However, we have observed that the expression
398 levels of Th1- and Th2-specific transcription factors as protein was not correlated with those as
399 mRNA (Fig. E1). Further, mRNA expression levels of transcription factors were not consistent
400 with the hyper-expression of Th2 cytokines in asthmatic patients (Fig. 1). Therefore, the present
401 findings demonstrating a differential T-bet expression pattern in purified peripheral and cord
402 blood $CD4^+$ T cell populations at the protein level, seems to be more convincing and more
403 importantly, consistent with the results of the Th1/Th2 differentiation experiment.
404 The importance of T-bet to Th1 differentiation has been reported. T cells derived from
405 T-bet-deficient mice showed defective Th1 differentiation [30], and accordingly, these mice
406 spontaneously manifested Th2-related disorders [31]. Ectopically-expressed T-bet induced IFN-γ
407 synthesis in Th2-polarized cells of murine and human origins [32, 33]. The expression of T-bet in T
408 cells is correlated with polarization of the murine Th1 phenotype [32], though the correlation has
409 been poorly confirmed in human T cells. Consistent with these previous investigations, our
410 present study clearly demonstrated that IFN-γ-producing activity in human $CD4^+$ T cells is
411 generally correlated with resting expression pattern of T-bet protein.
412 We also demonstrated that, like murine T cells, ectopically expressed T-bet induced IFN-γ
413 synthesis in human $CD4^+$ T cells even after stimulation culture under Th2-polarizing conditions.
414 Furthermore, the functional role of endogenously expressed T-bet in IFN-γ synthesis was clearly
415 demonstrated by employing RNAi technology. Consistently, Lametschwandtner et al. reported
416 that ectopic expression of T-bet in a human Th2 cell line derived from the skin of patients with
417 atopic dermatitis induced IFN-γ-producing activity [33]. Therefore, it is suggested that T-bet plays a