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, 1×10^6 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

258 composed of a $CD25^-CD44^+CD62L^+CD69^-CD127^+CCR7^+$ homogeneous population (Fig. 2B),
259 even though the expression levels of the positive molecules differed in individual subjects (data
260 not shown), suggesting that these cells are recognized as typical naïve T cells. IL-4Rα and
261 IL-12Rβ1 but not IL-12Rβ2 were similarly expressed on both cells. After stimulation culture
262 under Th1 and Th2 conditions, respective phenotypes were clearly induced from cord blood naïve
263 $CD4^+$ T cells (Fig. 2C). Th1 differentiation was also successfully induced, though peculiar Th2
264 differentiation occurred in peripheral blood naïve $CD4^+$ T cells. Thus, IFN-γ-producing cells were
265 significantly developed even under Th2-skewing conditions (Fig. 2C).
266
267 **Peripheral naïve $CD4^+$ T cells significantly express T-bet due to high accessibility of the**
268 **proximal promoter region in the T-bet gene**
269 In order to elucidate the reason for the distorted Th2 differentiation in human peripheral
270 $CD4^+$ T cells, the expression of Th1- and Th2-specific transcription factors in peripheral and cord
271 blood naïve/Th1/Th2 cells was comparatively analyzed by western blot. As shown in Fig. 3A,
272 GATA-3 expression was hardly observed in both peripheral and cord blood naive $CD4^+$ T cells
273 and slightly induced upon stimulation, whereas it was markedly up-regulated by Th2
274 differentiation. The expression of c-Maf was relatively stable. In contrast, STAT4 expression was
275 clearly up-regulated after Th1 differentiation in both peripheral and cord blood $CD4^+$ T cells. In
276 cord blood-derived cells, T-bet was also inducible and preferentially expressed in Th1 cells.
277 However, peripheral $CD4^+$ T cells displayed an interesting expression pattern of this transcription
278 factor. Thus, the expression of T-bet was not only equivalent in Th1 and Th2 cells, but was also
279 clearly detectable in naive $CD4^+$ T cells even without stimulation (Fig. 3A).
280 It has been demonstrated that T-bet down-regulated the transcriptional function of its
281 opposing transcription factor, GATA-3 [24]. Phosphorylation of a tyrosine residue in T-bet was
282 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

crucial role in expression of IFN-γ in human $CD4^+$ T cells even under a Th2-skewing state, such as in allergic asthma.

Nevertheless, peripheral naïve $CD4^+$ T cells failed to produce IFN-γ, regardless of significant expression of T-bet. In addition to the requirement of other transcription factors, such as NFAT, NFκB and AP-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 naïve $CD4^+$ 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 upon stimulation and significantly expressed also in naïve and Th2 cells at 24 h (Fig. 3A). However, the *de novo* synthesized T-bet did not contribute to the expression of IFN-γ (Fig. 2C). As the correct reason for the discrepancy is not clear, it is probably due to the difference in time course, since cytokine producing activity was determined in an earlier time point (6 h). In addition, our present findings suggest that the protein expression levels of transcription factors at the start of stimulation are crucial for inducible Th1/Th2 cytokine synthesis.

We and Lametschwandtner et al. demonstrated apparently contradictory findings. Thus, in addition to the strong promoting activity on IFN-γ, down-regulation of IL-4 and IL-13 by T-bet was observed in our present study, whereas Lametschwandtner et al. showed that IL-4 synthesis was not affected by ectopic expression of T-bet in human Th2 cells [33]. The reason for the contradiction is unclear, though our present study further demonstrated that T-bet-mediated down-regulation 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 up-regulated in T-bet-deficient mice [36]. Even though Th1 and Th2 cytokines counteract each other, down-regulation 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

442 expression, since Jurkat Tag cells failed to produce a detectable amount of IFN-γ, as well as IL-4,
443 IL-5 and IL-13 in the culture supernatant (less than 20 pg by 10^6 cells).

444 Nevertheless, Szabo et al. showed that T-bet failed to affect the promoter activity of
445 murine IL-4, even though IFN-γ promoter activity was augmented [30]. They used a reporter
446 construct containing -760 to +68 of the murine IL-4 promoter and murine EL-4 cells, while we
447 employed a human T leukemia cell line. Since the homology of the ~1 kb 5'-flanking regions of
448 the human and mouse IL-4 genes is ~60 %, the effect of T-bet on the IL-4 promoter may vary
449 among species. As T-bet-binding activity of the human and murine IL-4 and IL-13 gene
450 promoters, including the T-box-like region, has not been evaluated, further study will be needed
451 to identify the *cis*-regulatory elements responsible for T-bet-mediated down-regulation of IL-4
452 and IL-13 gene transcription.

453 Recently, the counteractive effects of T-bet on Th2-specfic transcription factors have been
454 investigated. GATA-3 expression in murine $CD4^+$ T cells was up-regulated in T-bet-/- mice and
455 contrary, suppressed by retroviral introduction of T-bet [37]. Our present study gained new insights
456 into the role of T-bet in GATA-3 expression in human $CD4^+$ T cells. Thus, the expression levels
457 of T-bet in GATA-3-low and -high populations in Th2 cells derived from peripheral blood $CD4^+$
458 T cells are equivalent. In addition, GATA-3 mRNA expression was minimally affected by ectopic
459 expression of T-bet in peripheral T cells and Jurkat Tag cells (Fig. 4C and 6A), suggesting that
460 T-bet does not directly affect GATA-3 expression in human T cells. As the expression of
461 GATA-3 was slightly enhanced by knockdown of T-bet (Fig. 5D), this is probably due to a
462 secondary effect caused by the relative polarization to Th2 phenotype.

463 The phosphorylation of T-bet was more potent in Th2 cells than Th1 cells in parallel with
464 the expression of GATA-3 (Fig. 3C). It has been reported that ITK is necessary for GATA-3
465 association and phosphoryolation of T-bet [24]. Therefore, the possibility that GATA-3 is required
466 for the interaction with ITK and resulting phosphorylation of T-bet is also suggested. As the

467 physiological meaning of hyper-phosphorylation of T-bet in Th2 cells is unclear in our study,
468 Hwang et al. showed that phosphorylated T-bet interferes with the binding of GATA-3 to its
469 target DNA [24]. Therefore, a negative contribution of T-bet, expressed and phosphorylated in Th2
470 cells derived from peripheral blood, to GATA-3-mediated Th2 cytokine synthesis is suggested.
471 In conclusion, T-bet expressed in human peripheral $CD4^+$ T cells plays a crucial role in
472 their IFN-γ-productivity even in Th2-skewing conditions *via* not only strong induction of IFN-γ
473 but also down-regulation of IL-4 and IL-13 at the transcription level. Hyper-expression of this
474 transcription factor in naïve $CD4^+$ T cells, due to high accessibility to the proximal T-bet
475 promoter region, may be one of the primary reasons for the lack of down-regulation of IFN-γ,
476 regardless of up-regulation of Th2 cytokines, in Th2-diseases including allergic asthma.
477

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479

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481

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583 **Legends for figures**

584

585 **Fig. 1.** Expression of Th1/Th2 cytokines and transcription factors in $CD4^+$ T cells of asthmatic
586 patients. Messenger RNA expression in freshly isolated peripheral $CD4^+$ T cells of asthmatic
587 patients and healthy donors was determined by quantitative real-time RT-PCR method. Each
588 dataset (plot) and mean (bar) are expressed as mRNA abundance normalized with GAPDH
589 expression (n = 16-38). P values were determined by unpaired t-test with Welch's correction.

590

591 **Fig. 2.** Th1 and Th2 cell differentiation from peripheral and cord blood naïve T cells.
592 $CD45RA^+CD4^+$ cells were purified from mononuclear cells in peripheral and cord blood of
593 healthy subjects and stained for CD4 and CD45RA (A). Cell surface markers on the peripheral
594 (plain line) and cord blood (bold line) $CD4^+CD45RA^+$ cells were stained with specific or isotype
595 control Ab (indicated as gray area) (B). After 7 to 10 days of stimulation culture under Th1- and
596 Th2-skewing conditions, peripheral and cord blood $CD4^+$ T cells were stimulated with 5 nM
597 PMA plus 1 μM ionomycin for 6 h and stained for intracellular IL-4 and IFN-γ (C). The
598 representative plots and means ± SEM of cytokine positive cell percentages from four separate
599 experiments are shown.

600

601 **Fig. 3.** Expression of transcription factors in T cells. Purified naïve $CD4^+$ T cells and
602 differentiated Th1 and Th2 cells from peripheral and cord blood of healthy subjects were left
603 unstimulated or stimulated with 0.1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 for 24 h. Expression
604 of transcription factors and actin in the resulting cells was analyzed by western blot. Protein
605 samples from 10^6 cells of individual groups were loaded and blotted on the same membrane (A).
606 GATA-3 and T-bet expression in Th2 cells from peripheral $CD4^+$ T cells was examined by
607 intracellular staining (B). WCL of Th1 and Th2 cells from peripheral $CD4^+$ T cells were

608 immunoprecipitated with anti-T-bet Ab. The resulting IP samples or WCL were blotted with
609 anti-phosphotyrosine, anti-T-bet and anti-GATA-3 Abs (C). The results shown are representative
610 of four separate experiments. REA assay against HaeIII sites at the T-bet gene was performed on
611 unstimulated naïve and Th1- and Th2-differentiated peripheral and cord blood T cells (D). Data
612 are expressed as means ± SEM of percent intact DNA compared with HaeIII-untreated control (n
613 = 4-6).
614
615 **Fig. 4.** Effect of T-bet on cytokine synthesis in T cells. T-bet-IRES-Venus (T-bet) and empty
616 IRES-Venus (Vector) expression cassette were introduced into cord blood naïve $CD4^+$ T cells of
617 healthy subjects by a lentivirus infection system during 7 to 10 days of stimulation culture under
618 Th2 conditions. The resulting cells were stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h
619 and stained for intracellular IL-4 and IFN-γ (A). The representative plots and means ± SEM of
620 cytokine-positive cell percentages in Venus-positive population from four separate experiments
621 are shown. IRES-Venus-combined GATA-3, c-Maf, STAT4 and T-bet expression cassette were
622 introduced into peripheral naïve $CD4^+$ T cells during stimulation culture under neutral conditions.
623 The resulting Venus-positive cells (B; M1 population) were purified and left unstimulated or
624 stimulated with 5 nM PMA plus 1 μM ionomycin for 6 h, and the expression of cytokine and
625 transcription factor mRNA was measured by quantitative real-time RT-PCR (C). Data are
626 expressed as means ± SEM of mRNA abundance normalized with GAPDH expression (n = 4).
627
628 **Fig. 5.** Knockdown effect of T-bet on human T cell differentiation. A serial expression cassette
629 containing U6 promoter-driven shRNA and EF promoter-driven Venus (A) was introduced into
630 peripheral naïve $CD4^+$ T cells of healthy subjects using lentivirus during 7 to 10 days of
631 stimulation culture under neutral conditions. The resulting Venus-positive population (B; M1
632 population) in T-bet (Bold line) and control (plain line) shRNA-introduced cells was purified.

633 Gray area indicates untransfected background. Expression of T-bet and/or GATA-3 in the
634 purified cells was determined by quantitative real-time RT-PCR (C) and western blot (D). The
635 purified cells were left unstimulated or stimulated with 0.1 μg/ml anti-CD3 plus 1 μg/ml
636 anti-CD28 for 24 h, and the concentrations of cytokines in the culture supernatant were measured
637 by ELISA (E).
638
639 **Fig. 6.** Effect of T-bet on cytokine mRNA expression and promoter activity in human T cells. (A)
640 Jurkat Tag cells were transfected with pMACS-Tbet or empty vector (10 μg each). After 48 h,
641 H-2^k-positive cells purified by a magnetic cell sorting system were left unstimulated or stimulated
642 with PMA (5 nM) plus ionomycin (1 μM) in the presence of 40 μg/ml anti-IFN-γ for 6 h, and the
643 expression of cytokine and transcription factor mRNA was determined by quantitative real-time
644 RT-PCR method. Data are expressed as means ± SEM of mRNA abundance normalized with
645 GAPDH expression (n = 4). (B) Cells were transfected with pEF-Tbet or empty vector in the
646 presence of IFN-γ-EGFP, IL-4-EGFP or IL-13-EGFP (10 μg each). At 24 h after transfection,
647 cells were stimulated in the presence of 40 μg/ml anti-IFN-γ for 16 h and the promoter activity
648 was detected as the fluorescence of synthetic EGFP measured by flow cytometry (n = 4).