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

420 Nevertheless, peripheral naïve $CD4^+$ T cells failed to produce IFN-γ, regardless of
421 significant expression of T-bet. In addition to the requirement of other transcription factors, such
422 as NFAT, NFκB and AP-1, IFN-γ gene transcription is strongly affected by T cell receptor
423 signaling-triggered epigenetic modification of its gene locus [34, 35]. Therefore, T-bet might hardly
424 associate with and activate the IFN-γ promoter in peripheral naïve $CD4^+$ T cells.

425 The expression of T-bet also seems to be regulated at the level of transcription with
426 epigenetic modification of its gene locus. Indeed, T-bet was strongly inducible upon stimulation
427 and significantly expressed also in naïve and Th2 cells at 24 h (Fig. 3A). However, the *de novo*
428 synthesized T-bet did not contribute to the expression of IFN-γ (Fig. 2C). As the correct reason
429 for the discrepancy is not clear, it is probably due to the difference in time course, since cytokine
430 producing activity was determined in an earlier time point (6 h). In addition, our present findings
431 suggest that the protein expression levels of transcription factors at the start of stimulation are
432 crucial for inducible Th1/Th2 cytokine synthesis.

433 We and Lametschwandtner et al. demonstrated apparently contradictory findings. Thus, in
434 addition to the strong promoting activity on IFN-γ, down-regulation of IL-4 and IL-13 by T-bet
435 was observed in our present study, whereas Lametschwandtner et al. showed that IL-4 synthesis
436 was not affected by ectopic expression of T-bet in human Th2 cells [33]. The reason for the
437 contradiction is unclear, though our present study further demonstrated that T-bet-mediated
438 down-regulation of IL-4 and IL-13 was achieved at the mRNA transcription level. In agreement
439 with our findings, IL-4 and IL-13 production by $CD4^+$ T cells was up-regulated in T-bet-deficient
440 mice [36]. Even though Th1 and Th2 cytokines counteract each other, down-regulation of IL-4 and
441 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

478 **Acknowledgements**

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).

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

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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