

Sustained T-bet expression confers polarized human T_H2 cells with T_H1-like cytokine production and migratory capacities

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Background: The transcription factor T-bet mediates IFN- γ production by T_H1 cells and suppresses T_H2 cytokine production when ectopically expressed in polarized murine T_H2 cells. Thus T-bet-mediated inhibition of T_H2 cytokine production might be beneficial for the treatment of allergic diseases like asthma or atopic dermatitis.

Objective: We sought to investigate the effects of ectopic T-bet expression in highly polarized human T_H2 cells obtained from skin biopsy specimens of patients with atopic dermatitis.

Methods: The cytokine production of T_H2 cells retrovirally transfected with a vector expressing human T-bet was determined by means of intracellular FACS staining and ELISA. The effects of T-bet transfection were analyzed at the mRNA level by means of real-time PCR and DNA microarrays and confirmed by using functional chemokine response assays.

Results: Transfection of T-bet into T_H2 cells induced high levels of IFN- γ and suppressed IL-5, but IL-2 and IL-4 production remained unchanged. T-bet transfection also induced IL-12R β 2 and CXCR3 expression on human T_H2 cells, whereas the IL-18 receptor was only induced as a consequence of T-bet-mediated increased responsiveness to IL-12. Furthermore, sustained T-bet expression in human T_H2 cells induced IL-2 production and decreased the secretion of IL-4. In addition, the chemokine receptor repertoire of these cells was changed toward a T_H1-like profile.

Conclusion: The combined switch in cytokine pattern and migratory potential of highly polarized human T_H2 cells mediated by T-bet might provide an additional advantage for the treatment of allergic diseases. (J Allergy Clin Immunol 2004;113:987-94.)

Key words: T_H1/T_H2 cells, T-bet, cytokines, chemokines, allergy, atopic dermatitis

CD4⁺ T_H cells can be divided into different subsets according to their cytokine secretion profiles.¹ In particular, human T_H1 cells produce IFN- γ and IL-2, whereas T_H2 cells produce IL-4, IL-5, and IL-13 as key signature cytokines. IL-12 plays a key role in the differentiation of

Abbreviations used

AD: Atopic dermatitis

GFP: Green fluorescent protein

HPRT: Hypoxanthine guanine phosphoribosyltransferase

IER: Immediate early response

T_H1 cells.² IL-12 receptor (IL-12R) β 2 expression is preferentially found on T_H1 cells, maintaining responsiveness to IL-12, whereas T_H2 cell development is accompanied by a loss of IL-12R β 2 expression. Typically, T_H1 cells also express IL-18 receptor (IL-18R) and migrate to inflammatory sites in response to CXCR3 and CCR5 ligands.³ Likewise, the chemokine receptors CCR3, CCR8, and CCR2 are preferentially expressed on T_H2 cells and have been associated with the generation of pathogenic T_H2 responses.^{3,4}

The restricted expression of cytokines by polarized T_H cell subsets arises from differential expression of transcription factors. GATA-3 is essential for expression of IL-5 and induces IL-4 and IL-13, whereas c-maf has a more specific role in IL-4 production.² In contrast, the transcription factor T-bet is specifically expressed in murine T_H1 cells and mediates IFN- γ production.⁵ Transfection of T-bet into polarized murine T_H2 cells diminishes IL-4 and IL-5 production,⁵ although T-bet expression is not necessarily incompatible with T_H2 cytokine production.⁶ Because T_H2 cytokines secreted by allergen-specific human T_H cells play a crucial role in the development and maintenance of allergic diseases, T-bet-mediated inhibition of T_H2 cytokine production might be beneficial for the treatment of allergic diseases, including asthma⁷ and atopic dermatitis (AD).

Here we show that prolonged ectopic T-bet expression of human T_H2 cells derived from the skin of patients with AD reverts the cytokine production and chemokine receptor expression profiles.

METHODS

T-cell isolation and retroviral infection

T_H cells derived from the skin of patients with AD and peripheral blood were isolated as previously described.⁸ For retroviral infection, T_H cells were restimulated with PHA and irradiated allogeneic PBMCs and transfected with a retroviral construct containing the cDNA of human T-bet followed by an IRES1 sequence and a green fluorescent protein (GFP) reporter gene or an IRES-GFP control

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Received for publication December 2, 2003; revised January 28, 2004; accepted for publication February 6, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.02.004

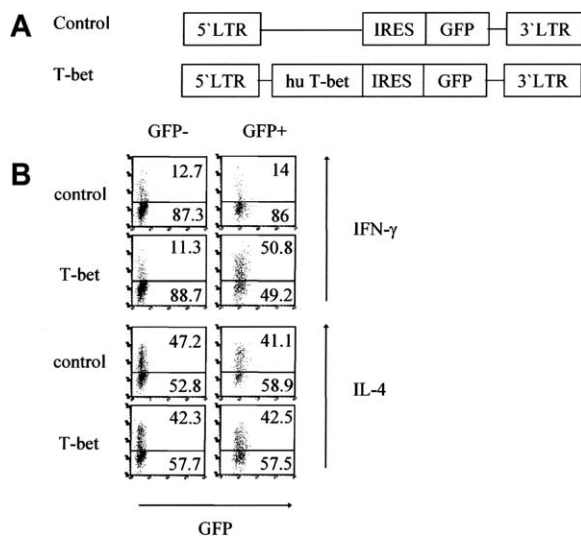


FIG 1. Ectopic expression of T-bet in human T_H2 cells derived from the skin of patients with AD. **A**, T_H2 cells were transfected with a retroviral construct coexpressing human T-bet and GFP (*T-bet*) or with GFP alone (*control*). **B**, Representative intracytoplasmic staining showing the percentages of IFN- γ - and IL-4-producing cells in the GFP-negative and GFP-positive fractions of control- and T-bet-transfected T_H2 cells.

alone, as previously described.⁵ Seven days after transfection, T_H cells were restimulated with PHA and allogeneic PBMCs and expanded with IL-2 for an additional 14 days.

ELISA and FACS analysis

Cytokine production was determined by using standard methods, as previously described.⁸ For ELISA, resting T_H cells were stimulated for 24 hours with 5 μ g/mL anti-CD3 mAb SPV-T3 (Zymed) bound to immunobeads (Irvine Scientific) and 5 μ g/mL soluble anti-CD28 mAb (PharMingen). Intracytoplasmic cytokine production was detected with APC-conjugated mAbs specific for IL-2, TNF- α (Caltag), IL-4, IL-5, or IFN- γ (PharMingen). All cytokine production measurements were performed in triplicate and are depicted as averages plus SD. Changes in cytokine production, discussed in the text as relevant, were statistically significant ($P \leq .05$, t test). For surface receptor expression, T_H cells were stained with phycoerythrin-conjugated mAbs (IL-18R and CXCR3, R&D; IL-12R β 2, PharMingen). FACS analysis was performed on viable cells by means of combined gating with forward light and side light scatter parameters and exclusion of propidium iodide-positive cells on a FACSCalibur cytometer by using CellQuest software (Becton Dickinson).

Microarray data analysis

T-bet-transfected and control T_H2 cells were restimulated with PHA and irradiated PBMCs for 6 cycles and sorted to a purity of greater than 99% on the basis of GFP expression. T_H cells were left either unstimulated or were stimulated with anti-CD3 and anti-CD28 mAbs for 4 hours. Total cellular RNA was prepared by using the Qiagen RNeasy Midi-Kit (Qiagen). RNA processing and hybridization to the Affymetrix gene chip HG-U133A (Affymetrix, www.affymetrix.com) was performed according to the manufacturer's recommendations. Microarray data were analyzed by using Genespring 5.0 software (Silicon Genetics). Gene expression values lower than zero were set to zero, and then all expression values were normalized per chip to the 50th percentile. Expression of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase

(*HPRT*) was almost equal in all samples, and thus all genes per chip were normalized to *HPRT*. For the purpose of this study, only fully annotated gene transcripts were considered. The analysis was further restricted to genes expressed with a minimum normalized value of 0.1 and flagged as present in at least 2 of 4 samples.

Real-time PCR

Total cellular RNA was prepared as above. PCR was performed on a GeneAmp 9600 Thermocycler (Perkin Elmer) by using gene-specific primers and probes (Table E1 in the Journal's Online Repository at www.mosby.com/jaci). Primers and probes were designed by using PrimerExpress Software (Applied Biosystems). Amplification was performed by using the following settings: 2 minutes at 50°C, 30 minutes at 60°C, and 5 minutes at 95°C, followed by 40 cycles of 20 seconds at 94°C and 1 minute at 62°C.

Analysis of intracellular calcium mobilization

T_H2 cells derived from skin of patients with AD were used 2 to 3 weeks after restimulation. Cells were loaded with Fluo-4/acetoxymethyl ester (Eubio-Molecular Probes) according to the manufacturer's instructions and plated in 50- μ L units at 4×10^5 cells/well (96-well Costar Viewplates). After 20 minutes' incubation at room temperature, Ca^{2+} mobilization was quantified by means of fluorescence reading on a FLEXstation (Molecular Devices) and analyzed by using SOFTMax Pro software.

Transwell migration assay

Aliquots of 5×10^6 /mL T_H cells (100 μ L/well) were plated in the top chambers of Transwell plates (Costar, catalog no. 3421), and lower chambers contained the chemokine in RPMI medium with 0.5% BSA and neutralizing anti-chemokine antibodies where indicated. Plates were incubated at 37°C for 3 hours, and specific migration was assessed by means of FACS analysis and expressed as a percentage of input cells.

RESULTS

T-bet modulates the expression of IFN- γ and IL-5, but not that of IL-4, in polarized human T_H2 cells independently of IL-12

Human T_H2 cell lines, producing large amounts of T_H2 cytokines but only little IFN- γ ,⁸ were isolated from skin lesions of patients with AD and transfected with a retrovirus coexpressing human T-bet and GFP (Fig 1, A). This method resulted in productive infection of up to 10% of the T_H2 cells, as assessed by means of GFP reporter gene expression. The T-bet-transfected T_H cell population was kept in culture together with the untransfected counterpart to avoid additional skewing from the autocrine release of T_H1 cytokines (eg, IFN- γ by transfected cells). Analysis of cytokine production at the single-cell level demonstrated that IFN- γ was specifically induced in the T-bet-transfected population but not in the cocultured, untransfected cell pool (Fig 1, B, and Fig 2), which indicates that IFN- γ production by T_H2 cells was a direct effect of T-bet. In addition, T-bet transfection reduced the percentage of IL-5-producing T_H cells but did not suppress the expression of IL-2 or IL-4. These effects were specific because the percentages of the different cytokine-expressing cells in the GFP-positive and GFP-negative fractions of T_H2 cells transfected with a control vector did not change. Furthermore, these results were not

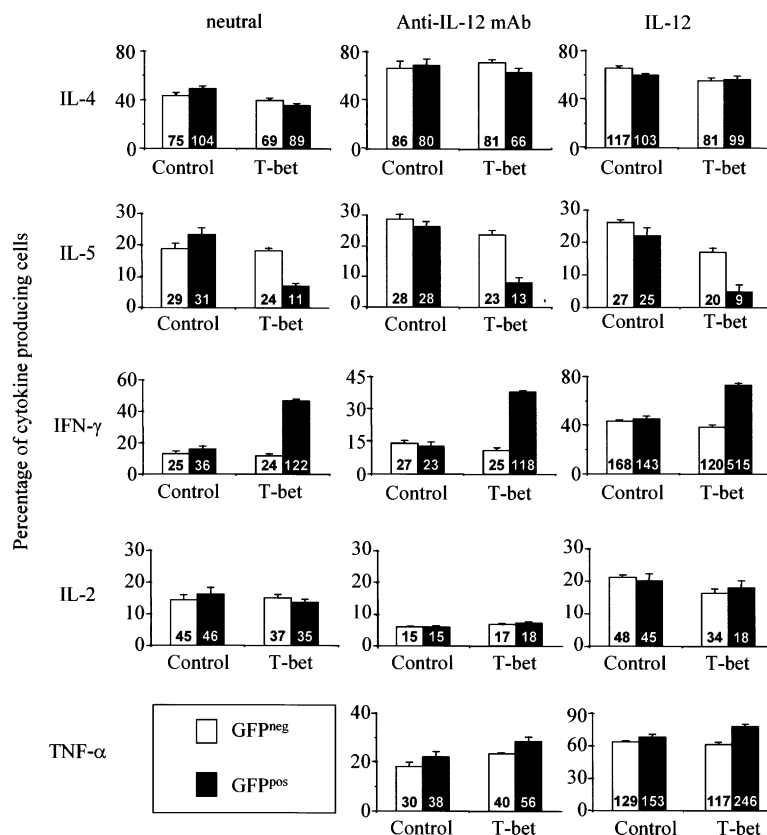


FIG 2. Cytokine production of T-bet–transfected T_H2 cells. T-bet– and control-transfected T_H2 cells were restimulated under neutral conditions or in the presence of 0.5 µg/mL anti-IL-12 mAb or 5 ng/mL recombinant IL-12, as indicated. Resting T_H cells were stimulated with anti-CD3 plus anti-CD28 mAbs and analyzed for cytokine production by means of intracellular FACS staining (gating on transfected: GFP^{pos}, filled bars; and untransfected: GFP^{neg}, open bars; populations as shown in Fig 1, B). Average fluorescence intensity values are indicated inside of each bar.

mediated by IL-12 produced by the feeder PBMC population because addition of exogenous IL-12 or neutralizing anti-IL-12 mAb provided similar results (Fig 2).

To exclude that IL-4 production in an allergic setting might not be reversible with T-bet (eg, because of altered transcription factor binding to the IL-4 promoter⁹ or genetic polymorphisms¹⁰), we tested the influence of T-bet on T_H cells from nonatopic individuals. T-bet strongly induced IFN-γ and repressed IL-5 production in T_H0 cells derived from peripheral blood of healthy donors but did not change IL-4 expression by these cells (Fig E1 in the Journal's Online Repository at www.mosby.com/jaci). Together, these results indicate that T-bet does not directly inhibit IL-4 production in human T_H cells.

T-bet induces expression of CXCR3 and IL-12Rβ2, but not of IL-18R

Next we examined the expression levels of IL-18R, IL-12Rβ2, and CXCR3 in T-bet–transfected T_H2 cells (Fig 3, A). CXCR3 and IL-18R expression was assessed in resting T_H cells, whereas IL-12Rβ2 expression was measured after stimulation with anti-CD3 and anti-CD28 mAbs. T-bet transfection in T_H2 cells induced CXCR3

and IL-12Rβ2 (Fig 3, A). In contrast, IL-18R induction required sustained T-bet expression over 2 cycles of stimulation with PHA and allogeneic PBMCs, and IL-18R induction was blocked by using addition of neutralizing anti-IL-12 mAbs (Fig 3, B). Together, these data indicate that IL-18R expression was due to the increased IL-12 responsiveness of T-bet–transfected T_H2 cells (Fig 3, B). Accordingly, addition of exogenous IL-12 to the system enhanced IL-18R expression on T-bet transfectants, whereas the induction of CXCR3 was independent of exogenous IL-12 or anti-IL-12 mAbs (Fig 3, B).

Sustained expression of T-bet in polarized human T_H2 cells induces T_H1 cytokines and represses T_H2 cytokines

The differential effects of T-bet on the expression of IL-12R and IL-18R prompted us to investigate whether sustained T-bet expression would induce a stronger T_H1 cytokine phenotype. To this end, T-bet– and control-transfected T_H2 cells were expanded over 6 cycles of restimulation and sorted to a purity of greater than 99% GFP-positive cells for determination of total cytokine production by means of ELISA. In addition to the

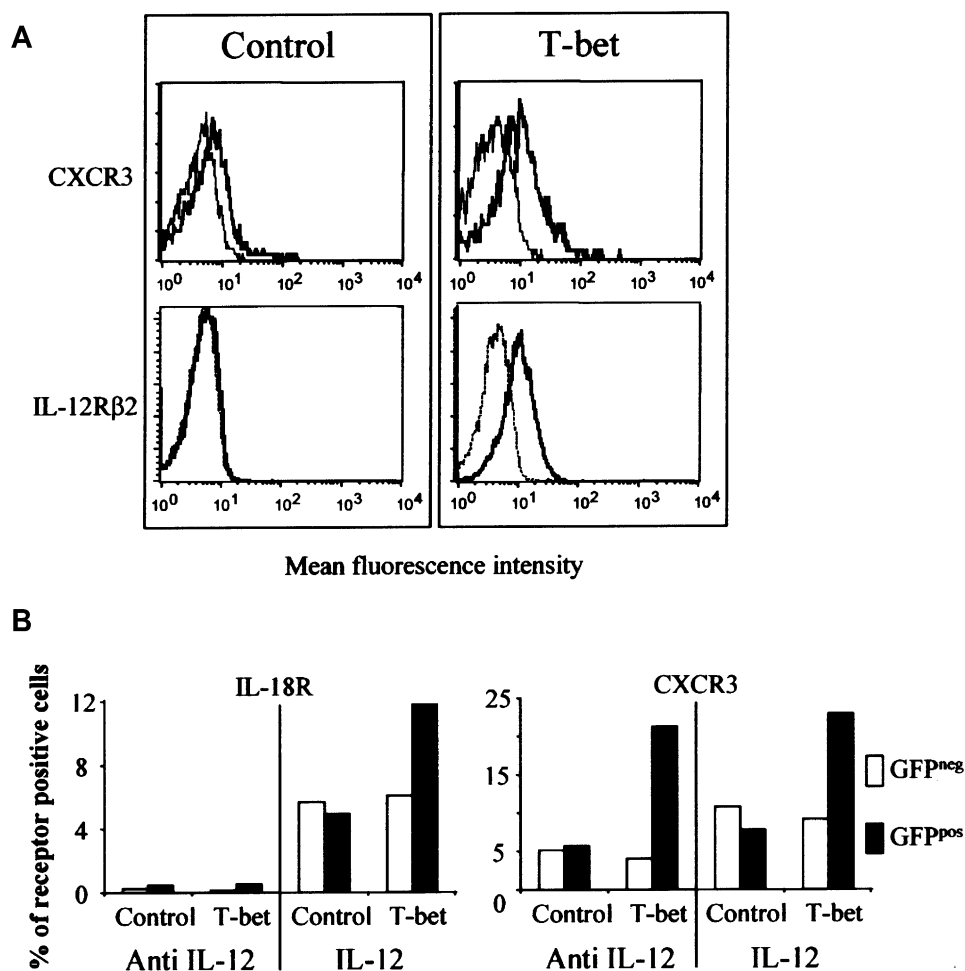


FIG 3. T-bet induces CXCR3 and IL-12Rβ2, but not IL-18R, on T_H2 cells. **A**, Expression of CXCR3 and IL-12Rβ2 on T_H2 cells 7 days after transfection with control or T-bet vectors was analyzed by using FACS on resting (CXCR3) or activated (IL-12Rβ2) cells. Dotted-slim lines represent isotype staining. **B**, IL-18R and CXCR3 expression on T_H2 cells transfected with indicated vectors after 2 cycles of stimulation in the presence of neutralizing anti-IL-12 mAbs or recombinant human IL-12.

enhancement of IFN- γ and the suppression of IL-5, sustained T-bet expression also repressed IL-4 and IL-13 production and induced the expression of IL-2 and TNF- α (Fig 4, A). These results were confirmed by means of intracellular FACS analysis. Sustained T-bet expression also reduced the percentage of IL-4-producing cells and simultaneously increased the percentage of IL-2 and TNF- α producers (Fig 4, A). In addition, the mRNA levels of the transcription factors c-maf and GATA-3, which regulate IL-4 and IL-5 production, were significantly lower in T-bet transfectants than in the vector control population (Fig 4, B).

Microarray analysis of genes modulated by sustained expression of T-bet in human T_H2 cells

T-bet or control transfected cells were stimulated (stim) with anti-CD3 and anti-CD28 mAb or left unstimulated (unstim), and subsequently, their mRNA transcripts were

analyzed with an Affymetrix U133A oligonucleotide microarray. Only defined gene transcripts that yielded clearly detectable signals on the microarray were included in the analysis. Because the most striking changes induced by T-bet expression in T_H2 cells were induction of IFN- γ and CXCR3 and repression of IL-5, we classified the microarray data into 3 categories: genes regulated like *IFNG* ($T\text{-bet}_{\text{stim}}/\text{control}_{\text{stim}} > 3$ and $T\text{-bet}_{\text{stim}}/T\text{-bet}_{\text{unstim}} > 3$; Fig 5, A), genes regulated like *CXCR3* ($T\text{-bet}_{\text{stim}}/\text{control}_{\text{stim}} > 3$ and $T\text{-bet}_{\text{unstim}}/\text{control}_{\text{unstim}} > 3$; Fig 5, B), and genes regulated like *IL5* ($\text{control}_{\text{stim}}/T\text{-bet}_{\text{stim}} > 3$ and $\text{control}_{\text{stim}}/\text{control}_{\text{unstim}} > 3$; Fig 5, C).

In agreement with ELISA and FACS data (Figs 3 and 4), IL-2, TNF- α , and IL-12Rβ2 were found to be strongly upregulated at the mRNA level (Fig 5, A). The adaptor proteins GADD45-β (Fig 5, A) and GADD45-γ (2-fold induction, not shown), which are involved in IL-12-dependent signal transduction, were clearly induced by T-bet. The immediate early response gene (*IER3*) has been demonstrated to protect cells from TNF- α - and

Fas-mediated cell death,¹¹ and thus the strong expression of *IER3* in T-bet transfectants (Fig 5, A) suggests a role for *IER3* counteracting the apoptotic effects of Fas ligand and TNF- α , which were both strongly upregulated in T-bet–transfected cells. CD30L/CD153 is another member of the TNF family that was upregulated by T-bet (Fig 5, A) and that has been recently reported to be implicated in the generation of antiviral T_H1 responses.¹²

After T-cell activation, T-bet transfectants downregulated the costimulatory molecule OX40 and the CC chemokine I-309/CCL1. Both molecules have been associated with T_H2 responses, and therefore their suppression further confirms the redirection of the T-bet–transfected T_H2 cells toward a T_H1 phenotype. Furthermore, independent of cell stimulation, T-bet transfection induced the expression of several surface molecules that have been reported to be preferentially expressed on T_H1 cells (IL-18R, IL-18RAcP, CD7,¹³ CD26,¹⁴ and CD27,¹⁵) or T_H1 functions, such as granzymes B and K.¹⁶ T-bet transfection also induced the expression of a series of chemokine receptors that are normally found on T_H1 cells^{3,17,18} (CXCR3, CCR5, CXCR6, and CCR1; Fig 5, B) and simultaneously repressed the expression of typically T_H2-related receptors,^{3,4} such as CCR3, CCR8, and CRTH2 (data not shown).

Sustained expression of T-bet in T_H2 cells results in a reversal of their chemokine receptor repertoire

Determination of mRNA levels by means of quantitative real-time PCR confirmed the induction of T_H1-associated chemokine receptors (CXCR3, CXCR6, and CCR5) and the concurrent suppression of T_H2-associated receptors (CRTH2, CCR3, and CCR8) in T-bet–transfected T_H2 cells (Fig 6, A). The relevance of these findings was proved in functional assays. The Ca²⁺ flux responses elicited by both CCR5 and CXCR6 ligands were only marginally higher in T-bet transfectants than in the control population (data not shown). However, the responses to the CXCR3 ligands IP-10/CXCL10 (not shown) and ITAC/CXCL11 were strongly increased in the T-bet–transfected cell population (Fig 6, B). Consequently, T-bet–transfected T_H2 cells displayed a strong CXCR3-dependent migratory response in vitro (Fig 6, C). The CXCR3-mediated migration of T-bet–transfected T_H2 cells was specific because it could be blocked by neutralizing anti-CXCL11 mAbs, and both control transfected and untransfected T_H2 cells did not display any significant response in the same transwell assay.

Together, these results indicate that ectopic T-bet expression not only modifies the cytokine secretion pattern of T_H2 cells but also reprograms the chemokine receptor pattern of these cells.

DISCUSSION

In this study we have shown that transfection of T-bet into polarized human T_H2 cells isolated from skin

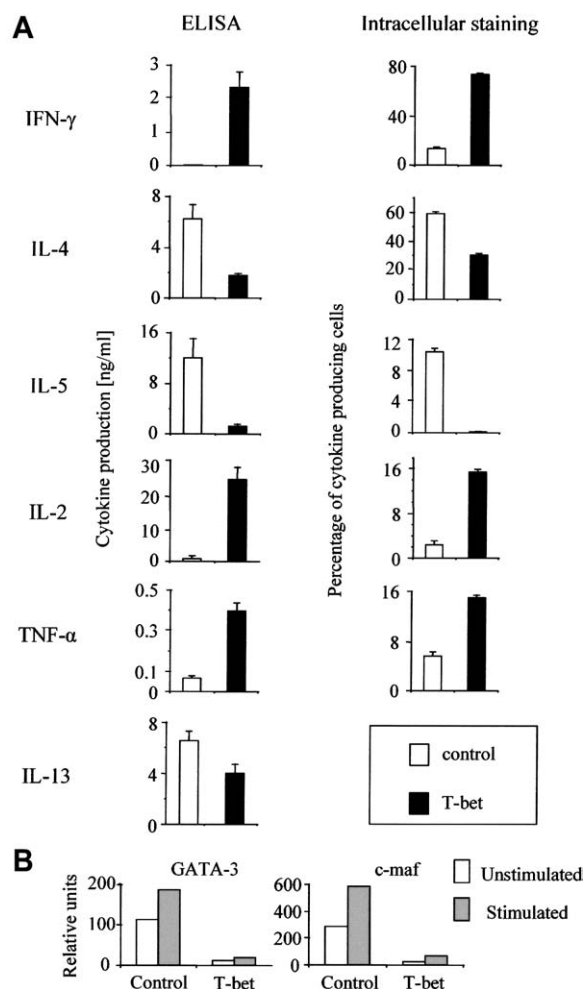


FIG 4. Sustained expression of T-bet in human T_H2 cells derived from the skin of patients with AD revert their cytokine production profile. **A**, T_H2 cells were infected as indicated, restimulated with PHA and irradiated PBMCs for 6 cycles, sorted to a purity of greater than 99% of GFP-positive T_H cells, and stimulated with anti-CD3 plus anti-CD28 mAbs. Cytokine production was determined by means of ELISA (overnight stimulation) or intracellular FACS staining (5 hours of stimulation). **B**, GATA-3 and c-maf expression levels were determined by means of quantitative real-time PCR. Bars represent average percentages of indicated transcripts equilibrated according to the housekeeping gene encoding hypoxanthine guanine phosphoribosyltransferase.

biopsy specimens of patients with AD induces IFN- γ and represses IL-5, but not IL-4, production. These results were independent of IL-12, and this was in contrast to IL-18R expression, which required T-bet–mediated IL-12 responsiveness. Sustained T-bet expression in human T_H2 cells not only suppressed IL-5 expression but also IL-4 and IL-13 expression. Thus far there is no evidence that T-bet binds directly to the promoter of IL-4 or IL-5. Thus the most likely mechanism by which T-bet mediated the suppression of these cytokines is through decreased activity of the T_H2 transcription factors c-maf and GATA-3. Indeed, c-maf and GATA-3 levels were decreased in T-bet–transfected T_H2 cells. In addition,

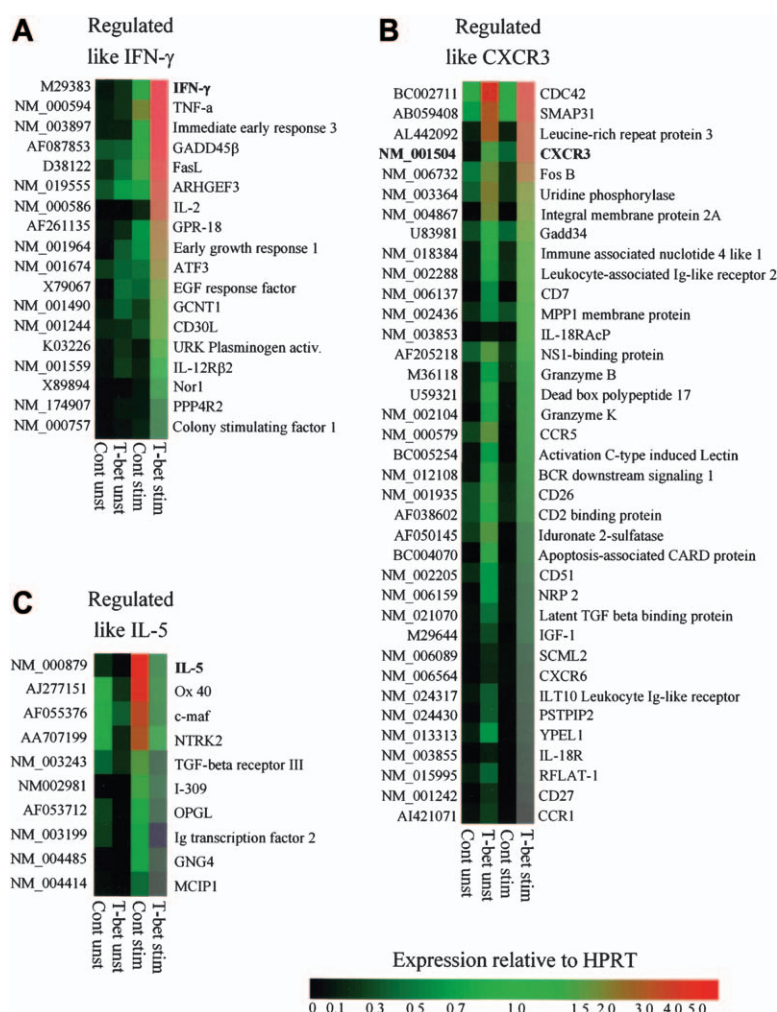


FIG 5. Microarray analysis of gene expression after sustained T-bet expression in human T_H2 cells. T_H2 cells were treated as described in Fig 4, and mRNA was analyzed by using an Affymetrix U133A oligonucleotide microarray chip. Genes were grouped into 3 categories: genes regulated like *IFNG* (**A**), genes regulated like *CXCR3* (**B**), and genes regulated like *IL5* (**C**). Relative expression was normalized to the expression of *HPRT* (defined as 1) and is represented by a color code. Genbank accession numbers for the transcripts are indicated in the left column.

sustained T-bet expression induced the production of IL-2 and TNF- α by T_H2 cells. This further conversion of the cytokine profile was not caused by the IFN- γ secreted by T-bet-transfected T_H2 cells because addition of anti-IFN- γ mAb did not have any effect (not shown). Furthermore, the expression of IFN- γ receptor 2, which has been described to be extinguished in T_H1 cells,¹⁹ was absent from T_H2 cells after sustained T-bet expression (data not shown), making a regulatory role of autocrine IFN- γ in this study highly unlikely. An important difference between our results and those reported in the murine system is the induction of IL-2 by sustained T-bet expression in human T_H2 cells. T-bet was found as a transcription factor that suppressed *IL2* mRNA.⁵ However, the observation that human T_H1 cells coexpress high amounts of IFN- γ , IL-2, and TNF- α (data not shown) suggest that T-bet might be permissive for IL-2 production

in human cells. This notion is supported by the fact that T-bet transfection also induced IL-2 production by Jurkat cells (Fig E2 in the Journal's Online Repository at www.mosby.com/jaci).

To extend the analysis on expression changes induced by sustained T-bet expression in human T_H2 cells, we performed a DNA microarray experiment. Thus far, studies investigating differential gene expression in human T_H1 and T_H2 cells have displayed a high degree of heterogeneity.²⁰⁻²² This can be partially attributed to the bimodal role of IL-12 used to polarize T_H cells, which has an additional effect on gene transcription.²⁰ Our results demonstrate that T-bet induces the expression of IL-12R β 2 chain and other factors, such as GADD45 β/γ , which is involved in IL-12 signaling. Thus the effects on gene transcription mediated by T-bet merge during T_H1 cell polarization with those mediated

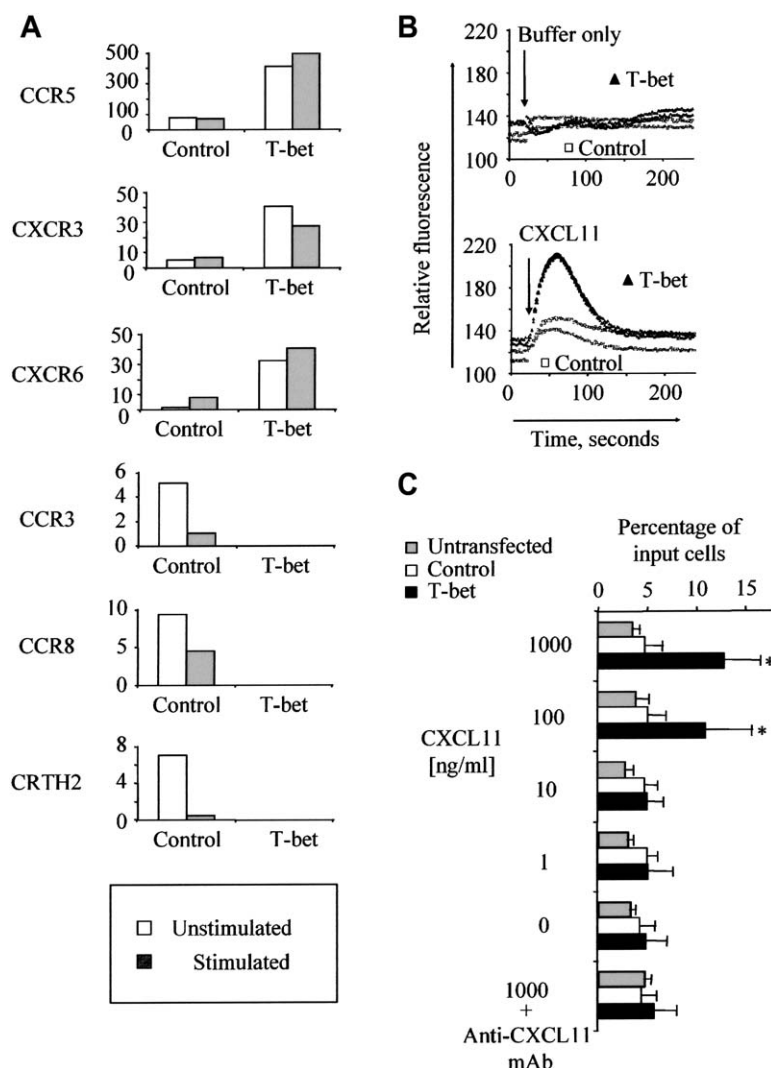


FIG 6. Sustained expression of T-bet in human T_H2 cells reverses their chemokine receptor expression pattern. **A**, The expression of CCR5, CXCR3, CXCR6, CCR3, CCR8, and CRTH2 was analyzed with quantitative real-time PCR, as in Fig 4. **B**, Intracellular calcium mobilization in T-bet- or control-transfected T_H2 cells by the CXCR3 ligand CXCL11. Two replica wells for each condition are shown. **C**, Dose-dependent transwell migration of untransfected or transfected (T-bet or control) T_H2 cells toward CXCL11. Bars represent average percentages plus SD of migrating of cells. Asterisks indicate significant migration ($P < .05$) in respect to corresponding null chemokine condition.

by the increased responsiveness to IL-12. In particular, we noted the induction of genes involved in 4 key features of T_H1 cell differentiation: genes involved in T_H1 polarization (eg, *IL12RB2*, *GADD45B/G*, *IL18R*, and *IL18RacP*), genes associated with T_H1 cytokine responses (*IFNG*, *IL2*, and *TNFA*), genes regulating cell death and apoptosis (Fas ligand, granzymes, and *IER3*), and genes controlling T_H1 migratory behavior (*CCR5*, *CXCR3*, and *CXCR6*). Simultaneous to the upregulation of T_H1 -associated chemokine receptors, T-bet downregulated the expression of T_H2 -related chemokine receptors, such as CCR3 and CCR8. In addition, T-bet also downregulated the expression of CRTH2, which is generally accepted as a T_H2 -associated marker.^{23,24} Interestingly, CCR4 was not downregulated (not shown)

in agreement with recent data demonstrating the coexpression of CCR4 and CXCR3,²⁵ particularly in skin-infiltrating T_H1 cell populations,²⁶ such as those involved in psoriasis.²⁷ This notion was further supported by the fact that T-bet-transfected T_H2 cells readily migrated in vitro in response to CXCR3 ligands (Fig 6, C) without losing CCR4 responsiveness (not shown).

Thus sustained T-bet expression in human T_H2 cells derived from the skin of patients with AD not only affects the cytokine production profile of these cells but also reverts their chemokine receptor repertoire, conferring a novel migratory potential.

We thank Frank Staedtler, Nicole Hartmann, and Markus Jaritz for microarray data analysis.

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