IL-12-induced T-bet expression and IFN\(\gamma\) release in lymphocytes from asthmatics—Role of MAPkinases ERK-1/-2, p38\(^{MAPK}\) and effect of dexamethasone

Andrea Koch\(^a\), Maria Raidl\(^a\), Michael Lux\(^a\), Katja Müller\(^a\), Hildegard Büning\(^b\), Sibille Humme\(^b\), Erland Erdmann\(^a\)

\(^a\)Department of Pneumology, University of Cologne, Medical Clinic III, Joseph-Stelzmann-Str. 9, 50924 Köln (Cologne), Germany

\(^b\)University of Cologne, Medical Clinic I and Center for Molecular Medicine Cologne, Germany

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**KEYWORDS**

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**Summary**

The transcription factor *T-box-expressed-in-T-cells* (T-bet) is required for Th1 lymphocyte differentiation, regulates the IL-12-induced expression of the Th1-specific cytokine IFN\(\gamma\), and may be dysregulated in asthmatics.

The modulatory role of extracellular signal-regulated kinase (ERK)-1/-2, p38mitogen-activated protein kinase (MAPK) and dexamethasone on IL-12 induced T-bet and IFN\(\gamma\) expression was assessed in peripheral blood lymphocytes of 10 atopic asthmatics and 10 nonatopic normals.

IFN\(\gamma\) production was dependent on phosphorylation of ERK-1/-2 and p38MAPK, as examined by PD098059, an inhibitor of the upstream activator of MAPK kinase (MKK-1), and SB203580, an inhibitor of p38MAPK. The inhibitory effect of PD098059 on IFN\(\gamma\) release was decreased in asthmatic T-cells compared with normals.

The IL-12-induced T-bet expression and the inhibitory effect of SB203580 were increased in asthmatic T-cells compared with normals.

Dexamethasone blocked the IL-12-induced T-bet expression in asthmatic T-cells completely and decreased IL-12-induced IFN\(\gamma\) release by \(\sim 50\%\), which occurred to the same extent in asthmatic and normal T-cells.

In conclusion, (1) p38MAPK-pathway rather than ERK-pathway may play a more basic role in the regulation of the increased T-bet expression in asthma, and (2) ERK- and p38MAPK-activation modulate IFN\(\gamma\) expression independently of T-bet and this regulatory role of
Introduction

Naive CD4+ T-lymphocytes, upon stimulation with antigens in the appropriate environment, differentiate into two classes of effector cells, T\textsubscript{H}1 and T\textsubscript{H}2. Increased numbers of CD4+ T-cells expressing IL-4 and IL-5 mRNA have been found in bronchoalveolar lavage (BAL)\textsuperscript{1,2} and in nasal and bronchial mucosa\textsuperscript{3,4} from allergic subjects. It has been demonstrated that peripheral blood lymphocytes from asthmatic subjects are primed for enhanced productions of IL-4 and IL-5 mRNA\textsuperscript{6} and GATA-3 DNA\textsuperscript{7} indicating a T\textsubscript{H}2-predominance in asthma.

IFN\textsubscript{\gamma}, a major cytokine from T\textsubscript{H}1 cells, directly suppresses IgE synthesis and inhibits the development toward T\textsubscript{H}2 cells.\textsuperscript{5,6} In young children with atopic disease, the reduction of IFN\textsubscript{\gamma} secretion was demonstrated.\textsuperscript{10,11} Infants whose cord blood cells had shown diminished IFN\textsubscript{\gamma} production in vitro were susceptible to the subsequent development of atopy.\textsuperscript{12} Furthermore, T\textsubscript{H}1-cells produce IFN\textsubscript{\gamma} to participate in cell-mediated immunity that controls infection by intracellular pathogens. The predominant factors that control T\textsubscript{H}1 differentiation are cytokines such as IL-12 to stimulate and IL-4 to inhibit T\textsubscript{H}1 differentiation.

The transcription factor T-box-expressed-in-T-cells (T-bet) has recently been described as a T\textsubscript{H}1-specific factor that plays a central role in T\textsubscript{H}1 differentiation.\textsuperscript{13} T-bet was described as a controller of T\textsubscript{H}1 development and was shown to have direct effects in IL-12 receptor \(\beta_2\) chain expression and IFN\textsubscript{\gamma} production.\textsuperscript{14-17} Additionally, transfection of T-bet into T\textsubscript{H}2 cells induced high levels of IFN\textsubscript{\gamma} and suppressed IL-5 and IL-4 generation.\textsuperscript{18} Finotto et al.\textsuperscript{19} described reduced numbers of cells staining for T-bet in bronchial biopsy specimens from asthmatic patients. Furthermore loss of T-bet results in increased airway hyperreactivity (AHR), an increased peribronchial and perivascular infiltration of eosinophils and lymphocytes, a greater amount of collagen around the airways and increased immunostaining of myofibroblasts in the airways of mice deficient in T-bet. So the authors speculate that a defect in T\textsubscript{H}1 development through a deficiency in T-bet might predispose to T\textsubscript{H}2-responses.

However, recent data argue against this model.\textsuperscript{20-22} Ylikoski et al.\textsuperscript{23} reported that in normal human cord blood IL-12 signaling is able to induce T-bet mRNA and protein expression during the early polarization of human CD4+ T-cells to T\textsubscript{H}1 direction and a likely mechanism behind it is IL-12 induced STAT1 tyrosine phosphorylation. Recently, Hwang et al.\textsuperscript{24} reported that T-bet represses T\textsubscript{H}2 lineage commitment through tyrosine kinase-mediated interaction between the two transcription factors that interferes with the binding of GATA-3 to its target DNA providing a novel function for tyrosine phosphorylation of T-bet and revealed that the role of T-bet tyrosine phosphorylation in repressing T\textsubscript{H}2 cytokines is independent of IFN\textsubscript{\gamma}. Moreover, Usui et al.\textsuperscript{20,25} could demonstrate in mice that naive T-cells tend toward T\textsubscript{H}1 differentiation through induction of GATA-3 and subsequent down-regulation of STAT4/IL-12R/\(\beta_2\) chain unless GATA-3 levels or function is regulated by T-bet indicating that the principal function of T-bet in developing T\textsubscript{H}1 cells is to negatively regulate GATA-3 rather than to positively regulate IFN\textsubscript{\gamma} gene. They also could shown that developing T\textsubscript{H}2 cells from T-bet \(-/-\) mice could differentiate into T\textsubscript{H}1 cells when STAT4 and IL-12R/\(\beta_2\) chain expression are maintained.\textsuperscript{25}

Regulation of transcription of immunomodulatory and inflammatory genes such as T-bet in response to extra-cellular stimuli is achieved by a variety of different signal transduction mechanisms. One of the most extensively studied signaling pathways into the nucleus involves the mitogen-activated protein (MAP) kinase (MAPK) cascade.\textsuperscript{26} At present there are at least three MAP kinase pathways which are defined according to the MAP kinase that is activated (a) the extracellular signal regulated protein kinase (ERK)-1 and -2 pathway, (b) the c-Jun N-terminal protein kinase (JNK) pathway and (c) the p38mitogen-activated protein kinase (p38MAPK) pathway. These are activated by different stimuli including growth factors, endotoxins, stress, pharmacological agonists and cytokines such as IL-12.\textsuperscript{27,28} A number of recent studies have demonstrated a major role for the components of the MAPK pathway in the regulation of innate and adaptive immune responses including T\textsubscript{H}1/T\textsubscript{H}2 differentiation\textsuperscript{29,30} but little is known about the modulatory role of the MAPKinasERK-1/-2 and p38MAPK on T-bet and IFN\textsubscript{\gamma} expression of human peripheral blood T-lymphocytes.

Corticosteroids constitute the most effective current anti-inflammatory therapy for acute and chronic forms of allergic diseases and asthma. Corticosteroids are highly effective in inhibiting the effector function of epithelial cells, eosinophils and T\textsubscript{H}2 cells and also in inducing T-cell apoptosis.\textsuperscript{31,32} These results are seemingly in conflict with a previous study by Cannarile et al.\textsuperscript{33} reporting that glucocorticoid-induced leucine zipper (GILZ), induced by dexamethasone, is involved in control of T lymphocyte activation and apoptosis using GILZ transgenic mice which overexpress GILZ in the T-cell lineage. Interestingly, those cells secrete more T\textsubscript{H}2 cytokines (IL-5, IL-13, IL-4) and produce less T\textsubscript{H}1 cytokines (IFN\textsubscript{\gamma}) than wild-type mice. Upregulation of T\textsubscript{H}2 cytokine expression correlated with increased expression of T\textsubscript{H}2 specific transcription factors (GATA-3) and decreased expression of T-bet indicating that GILZ is implicated in T\textsubscript{H}2 response development and may be one critical mechanism accounting for glucocorticoid immunomodulation.\textsuperscript{33}

In the present study, the potential role of the protein kinases ERK-1/-2 and p38MAPK and dexamethasone in modulating T-bet expression and IFN\textsubscript{\gamma} release in human peripheral T-lymphocytes were examined. As T-bet may be a potent inducer of the T\textsubscript{H}1 specific cytokine IFN\textsubscript{\gamma}, we were interested in measuring whether the amount of IFN\textsubscript{\gamma} release is impaired in asthma. The therapeutic benefit of dexamethasone on T-bet and IFN\textsubscript{\gamma} production seems to be critical.

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corresponds to their T-bet expression levels and compared normal and asthmatic individuals.

**Materials and methods**

**Subjects**

The study population consisted of 10 healthy non-atopic non-smoking volunteers (four males, six females; mean age: 30.7 ± 2.4 years), who had no history of asthma or of any respiratory disease with normal lung function and airway responsiveness to methacholine (PC20 > 16 mg/ml) and 10 mild atopic asthmatics (five males, five females; mean age: 28.8 ± 2.8 years) with an airway hyperresponsiveness to methacholine (PC20 < 8 mg/ml); positive PRICK- or RAST-tests for house dust mite etc. were included in this study (Table 1). All volunteers underwent peripheral blood separation after giving written informed consent. The study was approved by the Ethics Committee of the University of Cologne.

**Isolation of human peripheral blood lymphocytes**

A total of 50 ml of peripheral venous blood from 10 non-atopic normals and 10 atopic asthmatics were drawn into sterile syringes (Becton Dickinson, Franklin Lakes, NY, cat# 300296) by vein puncture with anticoagulant–citrate–dextrose (ACD) solution as anticoagulant at a final concentration of 10% (v/v). (ACD: 277 mM d-(+)-glucose; 142 mM fructose (ACD) solution as anticoagulant at a final concentration of 10% (v/v). The ACD-blood was prepared for sedimentation using 20 ml of ELO-HES (6-hydroxyethyl starch) and 5 ml of ACD (10%) per 25 ml of ACD-blood. The tubes were inversely mixed and air bubbles removed to prevent cell activation before leaving at room temperature for sedimentation of cells on a vibration free surface for approximately 60 min. The leukocyte-rich plasma fraction was centrifuged at 360g for 10 min and 4°C. The cell pellet was washed twice and then resuspended in about 2 ml of Hank’s balanced Salt Solution (HBSS, Sigma, St. Louis, MO, cat# H6648). and layered over 7 ml of Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden, cat# 17-1440-02) and centrifuged for 30 min at 360g and 18°C. The band of mononuclear cells (monocytes and lymphocytes) were collected from the interface and washed and resuspended three times in HBSS (Hanks buffered saline solution). After the third wash the cells were resuspended in RPMI 1640 (Sigma, cat# R-7638), supplemented with 10% fetal calf serum (FCS; Sigma, cat# N-4637), 2 mM L-glutamine, 100 U/ml penicillin, 100 µ/ml streptomycin (all from Sigma, Munich, Germany). Lymphocytes were separated from monocytes by depletion of monocytes by plastic adhererence seeding in a culture flasks in culture RPMI1640 medium in a humified atmosphere with 5% CO2 for 3 h. After 3 h, nonadherent cells (lymphocytes) were separated and used for further analysis. The proportion of CD3+-lymphocytes in this separated cell fraction was examined by FACS-analysis. The purified T-lymphocytes from mononuclear cell (MNC) samples were routinely > 98% CD3+ lymphocytes. Cells were > 85% viable, as assessed by trypan blue exclusion.

**FACS analysis**

Purified T-lymphocytes from mononuclear cells were incubated with CD14+ conjugated PE-Ab and CD3+ conjugated FITC-Ab and analyzed by flow cytometry in each test. Cells were initially gated on the basis of forward scatter and side scatter characteristics, with gates set to remove debris. Results were expressed as a percentage of cells exhibiting positive fluorescence (data not shown).

**Cell culture**

Peripheral blood T-lymphocytes were cultured at 37°C in a humified atmosphere with 5% CO2 in RPMI1640 medium (Sigma, Munich, Germany) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µ/ml streptomycin (all from Sigma, Munich, Germany). After 12 h cells were stimulated in fresh RPMI medium as above at 1 × 106 cells/ml for ELISA and with RPMI medium in the absence of FCS at 3 × 106 cells/ml for real-time-PCR and for Western blotting. CD3-purified mouse anti-human monoclonal antibody; (500 ng/ml; BD Becton Dickinson, Heidelberg, Germany; cat# 555336) and CD28-purified mouse anti-human monoclonal antibody (500 ng/ml; BD Biosciences; Germany, cat# 555725) were added 30 min before stimulation with IL-12 (10 ng/ml; R&D Systems, Wiesbaden, Germany; cat# 219-IL-005) for 72 h for ELISA and for 4 h for real-time-PCR. The ERK-1/-2 inhibitor, PD 089059 (10 µM; Calbiochem-Merck, Darmstadt, Germany, cat# 513000), the p38MAPK inhibitor SB 203580 (10 µM; Calbiochem-Merck, Germany) were present at the start of the stimulation. Table 1. Characteristics of non-atopic normals and atopic asthmatic subjects.

<table>
<thead>
<tr>
<th></th>
<th>Nonatopic normals</th>
<th>Atopic asthmatics</th>
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<tbody>
<tr>
<td>Female</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>30.7 ± 2.4</td>
<td>28.8 ± 2.8</td>
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<tr>
<td>FEV1 (% pred.)</td>
<td>102.6 ± 4.2</td>
<td>92.9 ± 4.6</td>
</tr>
<tr>
<td>IgE (U/l)</td>
<td>56.3 ± 4.7</td>
<td>284 ± 13.4</td>
</tr>
<tr>
<td>WBC (10^3/µl)</td>
<td>7.13 ± 0.5</td>
<td>6.45 ± 0.59</td>
</tr>
<tr>
<td>Lymphocytes (10^3/µl)</td>
<td>2.17 ± 0.1</td>
<td>1.58 ± 0.07</td>
</tr>
<tr>
<td>Lymphocytes (% WBC)</td>
<td>31.5 ± 1.9</td>
<td>25.3 ± 1.5</td>
</tr>
<tr>
<td>Eosinophils (10^3/µl)</td>
<td>0.16 ± 0.03</td>
<td>0.40 ± 0.14</td>
</tr>
<tr>
<td>Eosinophils (% WBC)</td>
<td>0.30 ± 0.5</td>
<td>6.50 ± 2.2</td>
</tr>
<tr>
<td>Monocytes (10^3/µl)</td>
<td>0.51 ± 0.05</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Monocytes (% WBC)</td>
<td>7.13 ± 0.5</td>
<td>6.60 ± 0.5</td>
</tr>
<tr>
<td>Neutrophils (10^3/µl)</td>
<td>4.35 ± 0.4</td>
<td>4.00 ± 0.5</td>
</tr>
<tr>
<td>Neutrophils (% WBC)</td>
<td>58.6 ± 2.6</td>
<td>60.8 ± 3.3</td>
</tr>
<tr>
<td>Basophils (10^3/µl)</td>
<td>0.03 ± 0.007</td>
<td>0.03 ± 0.007</td>
</tr>
<tr>
<td>Basophils (% WBC)</td>
<td>0.52 ± 0.1</td>
<td>0.57 ± 0.1</td>
</tr>
</tbody>
</table>

WBC, white blood cells; FEV1, forced expiratory volume in 1 s; IgE, immunoglobulin E. Data shown as mean ± SEM.
Darmstadt, Germany; cat# 559389) and dexamethasone (10^{-6} M; Sigma-Aldrich, Munich, Germany, cat# D-4902) were added 30 min before stimulation with IL-12. Drugs were dissolved in dimethyl sulfoxide (Me_{2}SO) or distilled water and were diluted to final concentrations of less than 0.1% (v/v). Me_{2}SO alone had no effect on expression or generation of IFN_{γ}, T-bet or EF-1α (data not shown). We have done time- and concentration-dependent experiments for all treatments used.

**Enzyme-linked immunosorbent Assays (ELISA) for IFN_{γ}**

IFN_{γ} was assayed using a quantitative sandwich enzyme immunoassay technique. 96-well plates (NUNC Inc) were coated overnight at room temperature with 100 µl of a monoclonal anti-human IFN_{γ} antibody (2 µg/ml) (R&D Systems; Wiesbaden, Germany; cat# MAB285). After washing with TBS-Tween-20®°, the antibody was blocked with PBS/1% BSA; 5% sucrose (Sigma, Munich, Germany) and 0.05% NaN_{3} (300 µl/well, Sigma, Munich, Germany) for 1 h. IFN_{γ} standards and plasmid standards were added to the plate for 2 h at room-temperature and washed with PBS-Tween-20° four times. A biotinylated secondary anti-human IFN_{γ} antibody (R&D Systems; cat# BAF285) non-diluted samples were added to the plate for 2 h at room-temperature and washed with PBS-Tween-20°. The cDNA was subsequently diluted to a final concentration of 10^{-6} cells. RNA was heat denatured at 95 °C for 60 min followed by 93 °C for 5 min. The cDNA was subsequently diluted to a final volume of 60 µl with nuclease-free water.

**RT-Real time PCR for T-bet and elongation factor (EF)—1α**

**RNA isolation and cDNA synthesis**

RNA of T-lymphocytes (3·10^6 cells) was extracted using Qiagen kit (RNeasy Mini kit, Qiagen, Hilden, Germany, cat# 74106), according to the manufacturer’s instructions and RNA was finally eluted in 30 µl nuclease-free water. The concentration and purity of RNA were assessed by spectrophotometry. 10 µg of RNA was treated with DNase (Promega Corporation, Madison, WI, USA; cat# M610A) at 37 °C for 15 min. After addition of 2 µl of stop solution (Promega Corporation, Madison, WI, USA cat# M199A) RNA was heat inactivated at 65 °C for 10 min. Reverse transcription of 0.5 µg of DNase treated RNA was performed using random hexamers (Promega Corporation, Madison, WI, USA; cat# C1181) and Omniscript RT kit (Qiagen, Hilden, Germany, cat# 205 111) according to the manufacturer’s instruction and samples were incubated at 37 °C for 60 min followed by 93 °C for 5 min. The cDNA was subsequently diluted to a final volume of 60 µl with nuclease-free water.

**Standard-plasmid construction**

We generated plasmid-standards to quantify the gene expression. Plasmids containing a gene region of the T-bet or EF-1α genes were created by PCR-mediated cloning. A backbone pBluescript SK-plasmid (Stratagene, La Jolla, CA, USA, cat# 212206) was linearized with BamHI and dephosphorilized. For the insert production the amplification primer pairs for the real-time PCR (see below) were used supplemented with a BamHI-restriction site at the 5′ end. With these primers a conventional 3-step PCR on a lymphocyte cDNA was performed. The obtained inserts were purified by a gel extraction and followed by a BamHI digest to obtain sticky ends. The inserts were cloned into the linearized plasmid-backbone. Subsequently we performed a functional test with the real-time PCR. The concentration of plasmid-DNA was assessed by spectrophotometry and the copy number was calculated. Standard curves for each gene were generated using 10-fold serial dilutions (10^2–10^5 copies/4 µl) including a no template control.

**Real-time PCR**

Our initial plan to use the expression level of GAPDH in normalizing the results of T-bet failed. These results were consistent with those of Hamalainen et al. demonstrating that the transcription of a constitutively expressed gene in human CD4+ T-cells may change during cell culture in vitro and this was most pronounced with β-actin (up to 11.4-fold) and GAPDH (up to 17.0-fold). In contrast the expression of EF-1α and UbCH5B remained constant during the entire culture periods. Therefore, EF-1α was used in this study to normalize the T-bet expression.

For real-time PCR, the following intron spanning primer pairs were used: T-bet forward primer: 5′-GAT CAT CAC CAA GCA GGG ACG-3′, T-bet reverse primer: 5′-TCC ACA CTG CAC CCA CTG GC-3′, with an amplification length of 154 bp. EF-1α forward primer: 5′-GGG ATG GAA AGT CAC CCG TA-3′, EF-1α reverse primer: 5′-GCC GTG TGG CAA TCC AAT-3′, with an amplification length of 461 bp (all from VBC-Genomics Bioscience Research GmbH, Vienna, Austria). Real time PCR reaction contained 4 µl cDNA, 0.5 µM forward and reverse primer for T-bet or EF-1α, 4 µl LC-FastStart DNA MasterplusSYBR Green I—mix (Roche Diagnostics GmbH, Mannheim, Germany cat# 03515885001) and was diluted with nuclease free water to a final volume of 20 µl. cDNA samples, the plasmid-standards and one no-template control were measured for each gene analysis. PCR amplification and quantification were performed in a LightCycler™ 1.5 instrument (Roche Diagnostics, GmbH, Mannheim, Germany). After an initial denaturation at 95 °C for 10 min, a three step PCR was performed for 40 cycles: (1) denaturation at 95 °C for 10 s; (2) annealing at 60 °C for 5 s and (3) extension at 72 °C for 7 s. Fluorescence was detected at the end of each extension step (SYBR Green format). Melting analysis was performed following template amplification as a specific control. Therefore, the amplified product was slowly heated (0.1 °C/s) from 65 to 95 °C and fluorescence was measured continuously. Data evaluation was performed using LightCycler® software version 3.5 according to the suppliers recommendations. Sample concentration was calculated using standard curve with plasmid DNA template for each gene. Relative gene expression was expressed as a ratio of T-bet copies to EF-1α copies.
Sodium dodecyl sulphate-gel electrophoresis and Western blotting

The status of T-bet and GAPDH were assessed by Western immunoblot analysis using antibodies that recognize T-bet and GAPDH proteins. Cell pellets were lysed with mammalian cell lysis kit (Sigma, Munich, Germany; cat# MCL1-1KT) and denatured proteins (30 μg) were subsequently separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 10% vertical slab gels and transferred to Hybond electrochemoluminescence (ECL) membranes (Amersham Biosciences, Freiburg, Germany). The nitrocellulose was incubated for 1 h at ~25°C in 25 mM Tris-buffered saline (TBS; pH 7.6; 150 mM NaCl) containing 0.1% Tween 20 (TBS-Tween 20), and 10% w/v nonfat milk and then for 2 h in TBS-Tween 20 containing 3% bovine serum albumin and primary antibodies directed against either T-bet (Santa Cruz CA, USA; cat# sc-21763), diluted 1:200 or GAPDH (Stressgen, Victoria, BC, Canada, cat# CSA-335) diluted 1:1000. Following these 10-min washes in TBS-Tween 20, the membranes were incubated for 60 min at ~25°C with rabbit anti-mouse HRP-conjugated immunoglobulin (Ig) G (Dako, Glostrup, Denmark, cat# 54549) diluted 1:2000 in TBS-Tween 20 supplemented with 1% nonfat milk for T-bet and also for GAPDH, and subsequently visualized by ECL. The relevant bands were quantified using a transilluminator with a light converter plate (coupled with video camera and computer), BioDocAnalyze 2.0 software (Biometra, Göttingen, Germany) normalized to the house-keeping protein GAPDH.

Data analysis

Data are represented as mean ± SEM. Data were analyzed by Student’s t-test for paired data or by one-way ANOVA/Bonferroni multiple comparison test. The null hypothesis was rejected when P < 0.05.

Results

IL-12-induced T-bet mRNA expression (real time PCR)—effect of PD 098059, SB 203580 and dexamethasone

There was no significant difference in basal (non-stimulated) and anti-CD3/anti-CD28-pretreated T-bet mRNA expression normalized to EF-1α in normal and asthmatic T-cells (T-bet/EF-1α ratio normals: basal: 0.0044 ± 0.0015; anti-CD3/anti-CD28: 0.01 ± 0.003, n = 10); T-bet/EF-1α ratio asthmatics: basal: 0.0041 ± 0.0009; anti-CD3/anti-CD28: 0.011 ± 0.0022; P > 0.05; n = 10; Fig. 1).

IL-12 (10 ng/ml) induced an increase in T-bet mRNA expression of anti-CD3/anti-CD28 pretreated T-cells from asthmatics compared with normals (0.033 ± 0.009 vs. 0.012 ± 0.0024; n = 10; P < 0.05). Stimulation with IL-12 (10 ng/ml) did not induce further T-bet mRNA expression of anti-CD3/anti-CD28 pretreated T-cells from normals (0.012 ± 0.0024 vs. 0.01 ± 0.003; n = 10; P > 0.05).

PD 098059 (10 μM) had no inhibitory effect on IL-12-induced increased T-bet mRNA expression in T-cells from asthmatics (0.033 ± 0.01; P > 0.05, vs. IL-12 alone; n = 10). However, the inhibition of the increased IL-12 induced T-bet mRNA expression in T-cells from asthmatics by SB 203580 (10 μM) was ~100% (0.0153 ± 0.007 vs. 0.033 ± 0.009; P < 0.05; n = 10), indicating that p38MAPK pathway may play a more basic role in the regulation of T-bet transcription in asthma.

Dexamethasone (10⁻⁶ M) reduced IL-12-induced T-bet mRNA expression in asthmatic T-cells nearly completely (T-bet/EF-1α ratio: 0.0099 ± 0.002 vs. 0.033 ± 0.009, P < 0.05; n = 10); (Fig. 1). PD 098059 and SB 203580 did not modulate the dexamethasone-induced inhibition of T-bet mRNA expression in T-cells from asthmatics and normals (Fig. 1).

IL-12-induced T-bet protein expression (Western blotting)—effect of PD 098059, SB 203580 and dexamethasone

Under basal (non-stimulated; data not shown) and under anti-CD3/anti-CD28 pre-treated conditions, expression of T-bet was detectable by Western blotting (Fig. 2) and was normalized to the house-keeping protein GAPDH (Fig. 2).

IL-12 (10 ng/ml) induced a significant increase in T-bet expression in asthmatic T-cells compared with normals (T-bet/GAPDH ratio: normals: 0.19 ± 0.03 vs. asthma: 0.57 ± 0.1; n = 10, P < 0.05).

PD 098059 (10 μM) had no inhibitory effect on IL-12-induced increased T-bet protein expression in asthmatic T-cells (0.76 ± 0.2 vs. 0.57 ± 0.1; P > 0.05, n = 10). However, the inhibition of the increased IL-12 induced T-bet protein expression in T-cells from asthmatics by SB 203580 (10 μM) was ~80% (0.21 ± 0.04 vs. 0.57 ± 0.1; P < 0.05; n = 10), indicating that p38MAPK pathway may play also a more basic role in the regulation of T-bet expression on post-transcriptional level in asthma.

Dexamethasone (10⁻⁶ M) reduced IL-12-induced T-bet protein expression in asthmatic T-cells nearly completely (T-bet/GAPDH ratio: 0.14 ± 0.04 vs. 0.57 ± 0.1, P < 0.05; n = 10); (Fig. 2). PD 098059 and SB 203580 did not modulate the Dexamethasone-induced inhibition of T-bet protein expression in T-cells from asthmatics and normals (Fig. 2).

IL-12-induced generation of IFNγ—effect of PD 098059, SB 203580 and dexamethasone

There was no difference in the basal and anti-CD3/anti-CD28-pretreated generation of IFNγ in human peripheral blood T-lymphocytes from adult atopic asthmatics (anti-CD3/anti-CD28: 305.3 ± 131.8 pg/ml; n = 10) compared with adult non-atopic normals (anti-CD3/anti-CD28: 273.2 ± 111.2 pg/ml; n = 10; graph not shown). IL-12 (10 ng/ml) induced an increase in IFNγ release in T-cells and this to the same extent in normals (714.9 ± 233.0 pg/ml; n = 10) and in the asthmatics (732.7 ± 354.7 pg/ml; n = 10; P > 0.05) (graph not shown).

PD 098059 (10 μM), an inhibitor of the upstream activator of ERK-1 and ERK-2, suppressed IL-12-induced IFNγ release by 41.1 ± 4.5% in T-cells from normals but only by 20.9 ± 6.3% in asthma (P < 0.05; n = 10; Fig. 3) indicating a diminished Tγ1-related ERK-modulation in asthma. SB 203580 (10 μM), an inhibitor of the α- and β1-isofrom of p38MAPK, was more effective on IFNγ release, causing an inhibition of
53.8 ± 7.4% in T-cells from normals and 64.3 ± 5.1% in T-cells from asthmatics without statistical significance between both groups (n = 10; P > 0.05; Fig. 3).

Dexamethasone (10⁻⁶ M) inhibited IL-12-induced IFNγ release by 34.5 ± 10.8% in T-cells from normals (n = 10) and by 52.1 ± 17.3% in asthmatics (n = 10; P > 0.05; Fig. 3). Interestingly, there was an additional inhibitory effect on IFNγ release when PD 098059 or SB 203580 were added in the presence of dexamethasone and this was statistically significant by SB 203580 in T-cells from normals (74.7 ± 7.1% vs. 34.5 ± 10.8%; P < 0.05; n = 10; Fig. 3).

Discussion

In this study, we could shown that the T-bet-specific transcription factor T-bet was constitutively expressed in peripheral blood T-lymphocytes without differences between non-atopic normals and atopic asthmatic volunteers. Furthermore, we demonstrated for the first time that the IL-12-induced T-bet mRNA- and protein expressions were significantly increased in asthmatic peripheral blood T-cells compared with normals.

Interestingly, the elevated T-bet expression seen in asthmatic but not in normal T-cells did not correspond to the increased IFNγ release seen in both groups. So we speculate that (1) IL-12 signaling is able to induce IFNγ production independently of T-bet mRNA and protein expression in Th1 cells and/or (2) IL-12 receptor β2 chain and/or STAT4 expressions could be down-regulated in asthmatic T-cells by elevated GATA-3 expression7,20,25 and/or (3) elevated GATA-3 expression in asthma7 may inhibit IFNγ expression20 (Fig. 4).

Our results are consistent with a study from Ylikoski et al., demonstrating that a high T-bet protein expression in human CD4⁺ T-cells from the cord blood of healthy
neonates does not necessarily correlate with IFNγ production.

Moreover, Usui et al.\textsuperscript{20,25} could shown that naïve T-bet−/−CD4+ T-cells from mice were able to produce IFNγ, and that the IFNγ promoter accessibility could proceed in the absence of T-bet under IL-12 and anti-IL-4 stimulation. This could be one explanation why in our study IL-12 stimulation was followed by an increase in IFNγ release but without increase in T-bet expression seen in normal T-cells, indicating that IL-12 did skew naïve Th cells towards Th1 although it did not augment T-bet.

Recently, Usui et al.\textsuperscript{25} could demonstrate that naïve T-cells from mice tend toward Th2 differentiation through induction of GATA-3 and subsequent down-regulation of STAT4/IL-12Rβ2 chain unless GATA-3 levels or function is regulated by T-bet. The authors therefore supposed that the principal function of T-bet in developing Th1 cells may be to negatively regulate GATA-3 rather than to positively regulate the IFNγ gene.

Demonstrating that T-bet is not essential for IL-12Rβ2 chain expression, Afkarian et al.\textsuperscript{21} suggest that T-bet has an indirect influence on such expression through its ability to obviate GATA-3 effects on STAT4. Indeed, Usui et al. have shown that STAT4 signaling can induce IL-12Rβ2 chain expression in the absence of T-bet and that GATA-3 down-regulation of the IL-12Rβ2 chain occurs through down-regulation of STAT4.\textsuperscript{20,25}

It is well known, that GATA-3 expression is increased in asthmatic T-cells.\textsuperscript{7} We speculate that elevated GATA-3 levels in asthma were followed by elevated T-bet expression seen in our study to further negatively regulate GATA-3 in asthma (Fig. 4). Indeed, Erpenbeck et al.\textsuperscript{22} reported that in healthy control subjects, the expressions of GATA-3, c-maf and T-bet in BAL cells were unchanged after saline or allergen challenge. In contrast, GATA-3, c-maf and T-bet were significantly upregulated after allergen challenge but not saline challenge in the asthmatic patients. This was surprisingly, because they expected a down-regulation of T-bet after allergen challenge in asthmatic T-cells, but the opposite was the case. The authors speculate that increased T-bet expression after allergen challenge in asthma might not only result from activated Th1 but also from activated Th2 cells and suggest that T-bet mRNA is not specific for a Th1-type inflammatory response.

Interestingly, Höhler et al.\textsuperscript{35} reported that the Th cell polarization and differentiation of Th1 cells may under the control of cytokines and transcription factors with mainly locally and genetically determined activity. Signature cytokines and cytokine signaling events of Th1 rather than Th2 cells may be genetically determined and implicate that Th2-associated diseases in humans such as asthma might be due to genetic variations in Th1 cytokine regulation via T-bet. Höhler et al. have done a twin study and found low variability of intrapair differences in monozygotic (MZ) twins compared with dizygotic (DZ) twins for IFNγ and T-bet, indicative of a strong genetic component in the expression of these Th1-associated proteins. There was in contrast little or no evidence for a genetic effect on Th2 differentiation, however. The fact that IL-12 stimulation was followed by an increase in T-bet expression of asthmatic T-cells but not in

![Figure 3](image-url)
normals, as seen in our study, highlights this genetically
determined mechanism further on TH1 level.

We speculate that the increased GATA-3 expression in
asthmatic T-cells were followed by a decrease in STAT-4
expression and this was followed by an increased down-
regulation of IL-12R/2 chain expression responsible for no
further increase in IFNγ release compared with normals.7,20,25

This could be one possible explanation why changes in T-bet
mRNA between naive and asthmatic T-cells did not correspond
to changes in IFNγ production seen in our study. Moreover,
elevated GATA-3 levels might downregulate IFNγ expression in
asthmatic T-cells while increasing IL-4 and IL-5 expression.36

We next investigated the role for phosphorylation of
extracellular signal-regulated kinases (ERKs) ERK-1, ERK-2
and p38MAPK by studying the ability of their respective
inhibitors PD 098059 and SB 203580 to regulate T-bet mRNA
transcription and protein expression and IFNγ release.

Evidence for this possibility has recently come from Agrawal
et al.37 who have shown that ERK1/C0 mice display a bias
toward TH1 type immune response with higher IFNγ production
and lower IL-5 production in MOG35-55-primed T-cells
indicating that ERK1 may modify the TH1/TH2 balance in
T-cells as a negative regulator of TH1 immune responses.

Whereas PD 098059 had no inhibitory effect on IL-12-
induced T-bet expression in asthmatic and normal T-cells
(Fig. 1) but decreased IFNγ release by ~40% in asthmatic and
to a lesser extent in normal T-cells (~20%) (Fig. 3), the
inhibitory effect of SB 203580 on IL-12 induced T-bet
mRNA and protein expression in asthmatic T-cells was
~100% (Figs. 1 and 2). In contrast there was no inhibitory
effect of SB 203580 on T-bet expression in normal T-cells but
SB 203580 decreased IFNγ release by ~60% and this without
differences between groups, indicating that (1) p38MAPK
pathway rather than ERK-pathway may play a more basic
role in the regulation of T-bet transcription and protein
expression in asthma and (2) that ERK- and p38MAPK
activation may modulate the IFNγ expression independently
of the T-bet signaling pathway and this regulatory role of
ERK-1/-2 on IFNγ release may impaired in asthma.

![Diagram](A)

**Figure 4**  Naive peripheral blood lymphocytes may tend toward
TH2 differentiation through induction of GATA-3 and subsequent
down-regulation of STAT4/IL-12R/2 chain unless GATA-3 levels
or function is regulated by T-bet20,25 (Panel A) indicating that
the principal function of T-bet in developing T_h1 cells is to
to negatively regulate GATA-3 rather than to positively regulate
IFNγ gene.

IL-12 (10 ng/ml) had no effect on T-bet expression but promotes
T_h1 response through an increase in IFNγ release. There was a
decrease in IL-12 induced IFNγ release but no effect on T-bet
expression in the presence of the specific ERK-inhibitor PD
098059 (10 μM), the specific p38MAPK-inhibitor SB 203580
(10 μM) and dexamethasone (DEX; 10⁻⁶ M).

Panel B: Stimulation with IL-12 (10 ng/ml) increased T-bet
expression in asthmatic T-cells and this could be inhibited
nearly completely in the presence of SB 203580 (10 μM) and DEX
(10⁻⁶ M). IL-12 induced an increase in IFNγ release in asthmatic
T-cells and this to the same extent as in normals.

In contrast PD 098059 (10 μM) did not modulate the elevated
T-bet expression in asthma. In the presence of SB 203580
(10 μM) and DEX (10⁻⁶ M) the IFNγ release was reduced and this
to the same extent as in normals. In contrast PD 098059 (10 μM)
modulated IFNγ release in asthma to a lesser extent compared
with normals. We speculate that increased GATA-3 levels in
asthmatic T-cells decreased STAT4 expression followed by a
decrease in IL-12 receptor β2 chain expression, and therefore
no additional increase in IFNγ release could be found compared
with normals.
This is consistent with previous findings by Duan et al., reporting that ERK activity in asthmatic mice was significantly higher as compared with normal mice. Moreover, they could show that a selective inhibitor of MAPK/ERK kinase U0126 suppressed OVA-induced airway hyperresponsiveness in inhaled methacholine in an in vivo mouse asthma model and reduced the serum levels of total IgE, lung tissue eosinophilia, airway mucus production and expression of VCAM-1 in lung tissues, implicating that inhibition of ERK-signaling pathway may have potential anti-inflammatory effects and therefore the authors speculate for a therapeutic potential for the treatment of allergic airway inflammation.

Corticosteroids are the most effective anti-inflammatory therapy for asthma. The predominant effect of corticosteroids is to switch off multiple inflammatory genes encoding cytokines and other mediators that have been activated during the inflammatory process. There is been considerable interest in how corticosteroids affect the T<sub>1</sub>-specific T-bet signal transduction pathway including the IFN<sub>γ</sub> release only by ~50% in asthmatic and normal T-cells indicating that (1) IFN<sub>γ</sub> is regulated not only by T-bet but also by additional pathways and (2) that the inhibitory effect of dexamethasone on T-bet expression is increased in asthma. In the context of our speculation, that an increase of dexamethasone on T-bet expression is increased in asthma seems more critically and needs to be further investigated. This is consistent with findings from Tsitoura et al., demonstrating that dexamethasone effectively suppressed human IgE production in vitro by IFN-gamma and IL-5: the role of soluble CD23 (s-CD23). This is consistent with findings from Tsitoura et al., demonstrating that dexamethasone effectively suppressed human IgE production in vitro by IFN-gamma and IL-5: the role of soluble CD23 (s-CD23). This is consistent with findings from Tsitoura et al., demonstrating that dexamethasone effectively suppressed human IgE production in vitro by IFN-gamma and IL-5: the role of soluble CD23 (s-CD23). This is consistent with findings from Tsitoura et al., demonstrating that dexamethasone effectively suppressed human IgE production in vitro by IFN-gamma and IL-5: the role of soluble CD23 (s-CD23).

In conclusion, the therapeutic benefit of inhibitors of the ERK- and p38MAPK pathways and of the anti-inflammatory drug dexamethasone on T<sub>1</sub>-specific T-bet expression and IFN<sub>γ</sub> release in asthma seems to be critical and needs to be further investigated.

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References


