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T-bet is induced by interferon- γ to mediate chemokine secretion and migration in human airway smooth muscle cells

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Singer CA. T-bet is induced by interferon- γ to mediate chemokine secretion and migration in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 300: L633–L641, 2011. First published January 14, 2011; doi:10.1152/ajplung.00163.2010.—An inappropriate balance between T-helper (Th)1 and Th2 cytokine production underlies inflammatory changes that result in airway disease. Expression of the T-box transcription factor T-bet regulates differentiation of Th cells and production of Th1 cytokines, particularly IFN γ . T-bet-deficient mice develop airway hyperreactivity, undergo airway remodeling, and exhibit defects in IFN γ production while overproducing Th2 cytokines. T-bet is also reduced in the airways of asthmatic patients, suggesting loss of T-bet expression or activity promotes development of inflammatory airway disease. We present novel data demonstrating T-bet expression is induced in human airway smooth muscle cells (ASMC) by IFN γ . This IFN γ -stimulated expression of T-bet is dependent on signaling through JAK2 and signal transducers and activators of transcription 1 (STAT1) and activates T-bet-dependent DNA binding activity. Expression of T-bet stimulates IFN γ -stimulated IFN γ expression, secretion, and promoter activity, while inhibiting IFN γ -stimulated release of chemokines including monocyte chemoattractant protein (MCP)-1/CCL2, regulated on activation normal T-expressed and secreted (RANTES)/CCL5, and eotaxin/CCL11. This is accompanied by changes in expression of the chemokine receptors CCR3 and IL12R β 2 and TNF α . T-bet expression also reduces chemotactic migration of ASMC in response to serum and PDGF, which contributes to airway hyperplasia. These results are the first to identify T-bet expression and activity in a structural cell of the lung and may provide new insights into therapeutic targets for inflammatory airway disease.

airway remodeling; inflammation; Janus-activated kinase; signal transducers and activators of transcription

IT HAS BEEN SUGGESTED THAT an imbalance between T-helper (Th)1/Th2 cytokine production underlies inflammatory changes in airway disease (21). Mature CD4⁺ Th1 and Th2 lymphocytes arise from naïve T-cell precursors that polarize upon exposure to specific cytokine signals, becoming divided into functional subsets based on the cytokines expressed (14). Th1 differentiation is driven by exposure to IL-12, IL-18, and IFN γ , and the cells continue to synthesize IL-2, IL-12, and IFN γ . Th2 cells develop under the influence of IL-4, synthesizing IL-4, IL-5, IL-10, and IL-13. IL-4 and IL-5 stimulate IgE synthesis and contribute to the recruitment and activation of eosinophils, while IFN γ decreases IgE production (31, 35, 46). Bronchoalveolar lavage (BAL) fluid from atopic asthmatic patients contains increased levels of IL-4 and IL-5 and decreased levels IFN γ (47, 56). Upon corticosteroid treatment airway hyperresponsiveness (AHR) and BAL fluid levels of

IL-4 and IL-5 are reduced, while an increasing number of cells express IFN γ (5, 40).

Th1/Th2 lineage commitment is directed by the expression and activity of multiple transcription factors, most notably T-bet and GATA-3, which transactivate promoters for Th1 or Th2 cytokines and their receptors. T-bet was initially isolated from a Th1 cDNA library and transcripts detected in lung, thymus, and spleen (52). Subsequently, T-bet was identified as a transcription factor expressed in Th1 cells transactivating the *IFNG* gene and inducing IFN γ production. Expression of T-bet into Th2 cells directs these cells to a Th1 phenotype by inducing IFN γ synthesis, while repressing IL-4 and IL-5 production (52) and increasing expression of chemokine receptors including IL-12R β 2 and CXCR3 (37). In the airways of asthmatic patients, T-bet expression is reduced, suggesting loss of T-bet expression may be associated with asthma (15, 29, 48). This idea is supported by findings in transgenic T-bet mice. T-bet knockout mice spontaneously develop airway hyperreactivity; undergo structural airway remodeling, as measured by increased basement membrane deposition of collagen; and produce increased amounts of IL-4 and IL-13 (15). In mice overexpressing T-bet, there is a shift toward a Th1 phenotype, alleviating some aspects of ovalbumin-induced airway remodeling including mucous production and eosinophilia (28). Based on these observations, the expression of T-bet in other airway cell types may be a major factor regulating the production of Th1 cytokines in the lung.

Airway smooth muscle (ASM) secretion of inflammatory mediators contributes to the recruitment and activation of immune cells in the airway and exacerbates airway hyperreactivity (45). Thus defining the mechanisms by which airway smooth muscle cells (ASMC) respond to inflammatory signals is of vital importance to better understand the role of smooth muscle in lung disease. We have undertaken a novel investigative approach in the following studies by examining expression, activity, and functions of T-bet in human ASMC. We present data demonstrating that the expression and activity of T-bet are induced by IFN γ in human ASMC. We further examine functions of T-bet on cytokine and chemokine secretion and receptor expression, as well as chemotactic migration of human ASMC. This work indicates that structural cells of the lung utilize mechanisms similar to those found in T cells to modulate Th1 cytokine and chemokine production, and functions of T-bet on ASMC may provide insights into new therapeutic targets inflammatory airway disease.

METHODS

Smooth muscle cell culture and treatments. Normal ASMC were obtained from Clonetics (San Diego, CA). All other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Cultures were maintained in a humidified 5% CO₂ atmosphere at 37°C in

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M199 media supplemented with 5% normal calf serum (NCS), 0.5 ng/ml epidermal growth factor, 5 μ g/ml insulin, and 2 ng/ml fibroblast growth factor. These cells have been screened by the supplier for human pathogens and the absence of nonsmooth muscle cell types. Further characterization of these cultures by our laboratory has been previously described (23, 51). Cultures from passages 4–8 were used for these experiments and upon confluence, growth arrested for 24 h in media supplemented with 0.1% NCS and growth factors. IFN γ (10 ng/ml, equivalent to 200 IU/ml; R&D Systems, Minneapolis, MN) stimulations took place at the times indicated. Selected cultures were treated with the 0.1% DMSO vehicle or the JAK2 inhibitor AG-490 (50 μ M; Calbiochem, San Diego, CA) for 15 min before and during IFN γ stimulation. For adenoviral transduction, confluent cultures were infected in 200 μ l of M199, 0.1% NCS for 60 min at the noted multiplicity of infection. Transduced cells were incubated in 0.1% NCS containing media for the duration of the experiment.

Generation of recombinant T-bet adenovirus. A cDNA clone expressing T-bet was isolated from human ASMC stimulated with 10 ng/ml IL-1 β , TNF α , and IFN γ for 20 h. Total RNA was extracted with TRIzol reagent (Invitrogen) and treated with DNase I (1U/ μ l) at 37°C for 15 min. RNA concentrations were quantified by measuring absorbance at 260 nm. First-strand cDNA synthesis was performed from 2 μ g total RNA using SuperScript II reverse transcriptase. T-bet specific oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) from the following sequence based on NM_013351: 5'-CGACGGCTACGGGAAGGTG-3' and 5'-TGT-CATCTGCTCAGTTGGGAAAAT-3'. T-bet was amplified using Platinum Taq polymerase (Invitrogen) at 94°C, 1 min; 57°C, 1.5 min; 72°C, 1 min followed by a final 72°C extension for 15 min. The resulting PCR product of 1.65 kb was cloned into the pTarget TA cloning vector (Promega, Madison, WI) and sequenced by dye-terminator sequencing at the Nevada Genomics Center. Recombinant adenovirus was generated using the AdEasy adenoviral vector system (Stratagene, La Jolla, CA) by digesting T-bet with *Sall* and *XhoI* for ligation into the corresponding sites of pAdTrack-CMV shuttle and recombination into pAdEasy. Successful T-bet/pAdEasy recombinants coexpress green fluorescent protein (GFP) behind a separate CMV promoter. Adenovirus was produced in a HEK293-derived viral packaging cell line and harvested, plaque-purified, and titered by an agarose overlay plaque assay as previously described (20). In all experiments, a recombinant adenovirus containing GFP but no transgene was used as a control for virus infection and designated AdGFP.

Gene expression analysis. The following oligonucleotides were designed to qualitatively examine T-bet expression in ASMC: 5'-ACTGGAGGTGTCGGGGAACTGA-3' and 5'-GCCGGGGCTGG-TACTTAT-GGAG-3' (Integrated DNA Technologies). Oligonucleotides specific for 18S rRNA were obtained from Ambion (Austin, TX). The reaction mixture contained 60 mM Tris-HCl (pH 8.5); 15 mM (NH $_4$) $_2$ SO $_4$; 1.5 mM MgCl $_2$; 0.25 mM dATP, dCTP, dGTP, dTTP; 10% DMSO; 50 μ M of each primer; 5 μ l template cDNA; and 2.5 units Taq polymerase. Amplification took place at 94°C, 30 sec; 66°C, 45 s; 72°C, 30 s. PCR products were visualized by ethidium bromide staining. Quantitative analysis of gene expression (qPCR) was evaluated using TaqMan assays and first-strand cDNA prepared from 2 μ g RNA diluted 1:5 as described above. Threshold cycle values from standard curves were used to quantify relative expression of specific RNA normalized to 18S rRNA. The specific TaqMan gene expression assays were as follows: T-bet (*TBX21*), Hs00203436; *GATA-3*, Hs00231122; IFN γ (*IFNG*), Hs00174143; *IL12RB2*, Hs00155486; *CCL11*, Hs00237013; and *TNF*, Hs00174128.

Western blot analysis. Whole cell extracts were prepared in buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1 mM EGTA, 1 mM EDTA, 5 mM NaF, 1 μ M leupeptin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride. Lysates were clarified by centrifugation at 10,000 g for 10 min at 4°C and protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as the standard. Total protein was separated by 10%

SDS-PAGE and transferred to nitrocellulose. Blots were blocked in Odyssey blocking buffer diluted 1:1 in PBS for 1 h (Licor Biosciences, Lincoln, NE) before incubation with primary antibody. The following antibodies were used in these studies: JAK1 (h-785); JAK2 (C-20); signal transducers and activators of transcription 1 (STAT1) p84/p91 (C-136); STAT3 (F-2), pSTAT1 (Tyr 101); pSTAT3 (Tyr 705); T-bet (4B10) from Santa Cruz Biotechnologies (Santa Cruz, CA); pJAK1 (Tyr1002/1023); and pJAK2 (Tyr 1007/1008) from Cell Signaling (Beverly, MA). For phospho-immunoblot analysis, immunoreactivity from phospho-specific blots in 15 μ g total protein was normalized to that from nonphosphospecific antibodies on the same membrane. For T-bet immunoblots, 50 μ g of total protein were used and for all blots secondary antibodies were conjugated to AlexaFluor 680 (Molecular Probes, Eugene, OR) or IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA) for fluorometric detection using an Odyssey infrared imaging system. All densitometric analyses took place within the linear range of the immunoreactive signal using Odyssey system software.

EMSA. Nuclear proteins were extracted according to standard protocols as previously described (4). Gel shift assays were performed using protocols found in the Gel Shift Assay Core System (Promega) (51) using 3.5 pmol double stranded oligonucleotides (Santa Cruz Biotechnologies, Santa Cruz, CA) end labeled with [γ - ^{32}]ATP and 4 μ g nuclear extract. Reactions were incubated at room temperature for 20 min and stopped with an appropriate volume of 10 $\times$ gel loading buffer containing 250 mM Tris-HCl (pH 7.5), 0.2% bromophenol blue, and 40% glycerol. Samples were loaded onto a 1.0 mm, 10 $\times$ 12 cm nondenaturing 4% acrylamide gels and electrophoresed at 100 V. Gels were dried overnight at room temperature between cellophane sheets and exposed to a phosphor-imager screen.

Promoter-reporter assays. Human ASMC were transiently transfected with luciferase reporter constructs to examine *IFNG* promoter activity using a modified CaPO $_4$ protocol (51). Twenty-four hours before transfection, cells were dispersed in 12-well plates at a density of 40,000 cells/well. The following day, normal growth media were removed and replaced with M199 containing 6% MBS, a modified bovine serum. The mouse *IFNG* promoter cloned into the pGL3 luciferase vector (Promega) was provided by M. J. Townsend (Harvard Medical School, Boston, MA), and an empty pGL3 vector used as the control. The DNA precipitate was prepared by addition of 5 μ g of pGL3 firefly luciferase DNA and 100 ng of pRL *Renilla* luciferase DNA to 2 $\times$ N,N-bis (2-hydroxyethyl)-2-aminoethane-sulfonic acid buffered saline, pH 6.95, and 0.125 M CaCl $_2$. Cultures were maintained for 4 h in a humidified 3% CO $_2$ atmosphere at 37°C to increase transfection efficiency. After being washed thoroughly in PBS, cultures were returned to a 5% CO $_2$ atmosphere and maintained in M199 containing 0.1% FBS and growth factors for 36 h before treatment. The Dual-Luciferase Reporter Assay System (Promega) was used to evaluate firefly and *Renilla* luciferase activity simultaneously from the same sample. Background luminescence was subtracted from non-transfected control cultures, and transfection efficiency was determined by normalizing firefly luciferase activity to *Renilla* activity in a Zylux Sirius model luminometer (Zylux, Oak Ridge, TN).

Small interfering RNA transfection. T-bet-specific small interfering (si)RNA sequences were purchased from Ambion and targeted human T-bet exons with the following sequences: Exon 6 (siRNA 1), 5'-GGAGCUUUUUGCA-UCUAGUTT-3'; exon 3 (siRNA 2), 5'-CCCAGAUGAUUGUGCUCCATT-3'; and exon 5 (siRNA 3), 5'-GGAUUCCGGGAGAACUUUGTT-3'. A nonbinding sequence was used as a negative control siRNA. After transduction with AdTBET for 24 h, media were removed and 30 nM siRNA were transfected using the siPORT NeoFX reagent (Ambion) in Opti-MEM (Invitrogen) and cells were maintained in 0.1% NCS containing M199 media. Assays were then performed 48 h following transfection.

Luminex and ELISA. Chemokine and cytokine secretion was measured by Luminex assay. Fifty microliters of sample were analyzed by Millipore (Billerica, MA) on the Luminex 100 using the Beadlyte

human multicytokine detection system according to the manufacturer's protocols. IFN γ ELISA assays were performed according to the manufacturer's protocols (R&D Systems, Minneapolis, MN).

Migration assays. Smooth muscle cell migration was assayed as previously described using a modified Boyden chamber with the following exceptions (11, 22). After adenoviral infection, 5.0×10^4 cells were plated on the upper side of the collagen-treated polycarbonate membrane (8.0- μ m pore; Costar, Cambridge, MA) and 1% serum or 10 ng/ml PDGF were added as a chemoattractant in the lower chamber. After 5 h, cells that migrated to the lower face of the membrane were fixed with 3.7% formaldehyde and stained with DifQuik Wright-Giemsa solution. The number of cells on the lower face of the filter was counted in seven fields under 10 $\times$ magnification.

Statistical analysis. Statistical analysis of multiple groups was performed by one-way ANOVA followed by post hoc testing with the Student-Newman-Keuls method using SigmaStat software (Jandel Scientific, San Rafael, CA). In selected instances, Student's *t*-tests were used to compare two groups.

RESULTS

T-bet expression is induced by IFN γ in human ASMC. To begin to elucidate functional consequences of T-bet expression in ASM, we examined T-bet expression in human ASMC. Figure 1A, *left*, shows that while T-bet mRNA is not detected at basal levels in human ASMC, treatment with 10 ng/ml IFN γ for 24 h induces T-bet mRNA expression. T-bet protein expression was confirmed by Western blot analysis in Fig. 1B.

We also determined that treatment with other Th1 polarizing cytokines, including IL-12 and IFN α , was also capable of up-regulating T-bet expression in human ASMC (Fig. 1A, *right*). The time course of induction of T-bet was further investigated using qPCR (Fig. 1C). Treatment with IFN γ produces a transient 2.4-fold increase in T-bet mRNA expression that peaks within 4 h and returns to basal levels. This is followed by a prolonged increase in expression that is 6.7-fold above basal levels after 24 h of IFN γ exposure. This increase in T-bet expression is seen with up to 48 h of IFN γ stimulation (data not shown). The biphasic increase in T-bet expression is likely due to a positive feedback loop by which IFN γ -stimulation of T-bet expression further stimulates IFN γ secretion and thus continues to stimulate increases in T-bet expression. Moreover, T-bet expression is accompanied by corresponding downregulation of GATA-3 mRNA expression, which acts as an opposing transcription factor during T-cell differentiation.

IFN γ -stimulation of T-bet-dependent DNA binding in human ASMC. To determine whether induction of T-bet expression in human ASMC correlated with functional protein, we examined T-bet-dependent DNA binding using EMSA. Representative gels from these experiments are shown in Fig. 1D. In the absence of stimulus (*lane 1*), we did not observe binding of nuclear proteins to an oligonucleotide corresponding to the T-bet consensus DNA binding site. Treatment with IFN γ for 2 h increased nuclear protein binding of the T-bet oligonucleo-

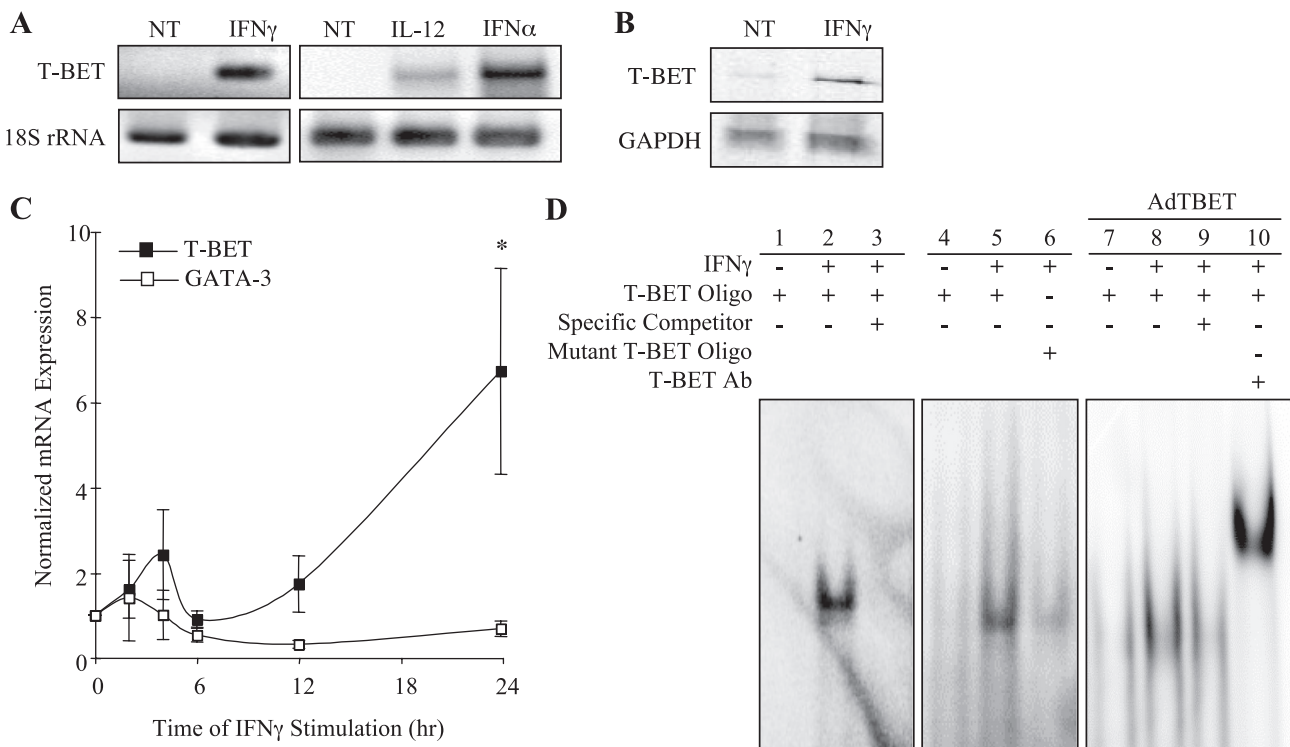


Fig. 1. T-bet expression and DNA binding activity are induced in human airway smooth muscle cells (ASMC). A: PCR products using T-bet-specific (top; 418 bp) and 18S rRNA primers (bottom; 324 bp) in human ASMC treated with 10 ng/ml IFN γ for 24 h, 25 ng/ml IL-12 or 500 U/ml IFN α for 48 h. Control cells were not treated (NT). B: immunoblot analysis of T-bet protein expression (top; 62 kDa) compared with GAPDH (bottom; 37 kDa) in IFN γ -treated ASMC. C: T-bet and GATA-3 expression detected by qPCR in cultures treated at the times indicated with 10 ng/ml IFN γ . Expression is normalized to 18S rRNA in all samples; $n = 3-4 \pm$ SE. * $P < 0.05$, significant difference from basal levels or GATA-3 expression. D: EMSA were performed using 4 μ g nuclear extracts from human ASMC cells treated with 10 ng/ml IFN γ for 2 h in the presence of ^{32} P-labeled T-bet consensus oligonucleotide. Excess unlabeled T-bet oligonucleotide was added to the reaction as a specific binding competitor in lane 3, and a mutated non-DNA binding T-bet oligonucleotide was added in lane 6. Human ASMC were transduced with AdTBET at 20 multiplicity of infection (MOI) for 3 days in lanes 7-10. Supershift was performed in lane 10 by preincubation with 5 μ l T-BET antibody. Representative gels are shown from $n = 3-5$.

tide (lane 2). This binding was ablated in the presence of excess unlabeled T-bet oligonucleotide (lane 3). We did not observe any differences in DNA binding with either 2 or 4 h of IFN γ treatment (data not shown), even though transient T-bet mRNA expression levels peak after 4 h. We further examined binding specificity with a labeled T-bet oligonucleotide in which the reported DNA binding site has been mutated, resulting in a dramatic reduction in T-bet DNA binding (lane 6). Similar results were observed in human ASMC transduced with AdTBET, where IFN γ -stimulated T-bet-specific DNA binding (lane 8) was abolished in the presence of excess unlabeled T-bet oligonucleotide as a specific competitor (lane 9). Addition of a T-bet-specific antibody to the reaction also results in a mobility supershift (lane 10). Taken together, the experiments demonstrate that increased expression of T-bet results in T-bet-dependent DNA binding.

T-bet expression is mediated by JAK/STAT signaling in human ASMC. Interferons transduce signals, in part, through the membrane-associated JAK and subsequent phosphorylation of various STAT proteins. In T cells, the induction of T-bet by IFN γ is dependent on JAK1 and JAK2 activation of STAT1 (1, 52). The contribution of JAK-mediated STAT1 phosphorylation to induction of T-bet expression in human ASMC was examined by phospho-immunoblot analysis. In initial experiments, IFN γ treatment resulted in time-dependent stimulation of JAK1/JAK2 and STAT1/STAT3 tyrosine phosphorylation with maximal phosphorylation observed within 15 min (data not shown). Pretreatment with 50 μ M AG-490, a JAK2 inhibitor, significantly reduced IFN γ -stimulated phosphorylation of JAK2 and STAT1 but not STAT3 shown as representative immunoblots in Fig. 2A and quantified by densitometry in Fig. 2B. This concentration of AG-490 was chosen because it has been demonstrated to block PDGF-dependent stimulation of JAK2 activity in ASMC (50) with no apparent effects on JAK1. Treatment with AG-490 also blocks induction of T-bet expression by IFN γ , as determined by qPCR analysis (Fig. 2C). The results demonstrate that IFN γ does stimulate upstream JAK2/STAT1 signaling pathways, as recently described (9), and that JAK2/STAT1 activity mediates T-bet expression in human ASMC.

T-bet expression stimulates IFN γ production. To facilitate the study of T-bet, a cDNA clone was isolated from human ASMC that encodes a protein identical to the human T-bet reference sequence (NP_037483). This cDNA was used to construct a T-bet expressing adenovirus (AdTBET). Protein expression levels of T-bet with AdTBET transduction increase proportionately with multiplicity of infection (Fig. 3A). Functions of T-bet in human ASMC were analyzed by examining the effect of AdTBET on IFN γ mRNA expression by qPCR (Fig. 3B). In cultures infected with the control AdGFP virus, IFN γ stimulation produced a 2.1-fold increase in IFN γ mRNA expression. Expression of AdTBET stimulated both basal and IFN γ -stimulated IFN γ mRNA expression by 8.6- and 18.9-fold, respectively. To correlate these changes in IFN γ expression with IFN γ secretion, we measured IFN γ by ELISA (Fig. 3C). In nontreated cells, IFN γ secretion was not detected. However, IFN γ was detected after 48 h in both noninfected and AdGFP cells at concentrations of 7.25 and 6.98 pg/ml, respectively, which is near the minimum detection level of the assay. This indicates that adenoviral expression itself does not affect IFN γ secretion, while expression of AdTBET signifi-

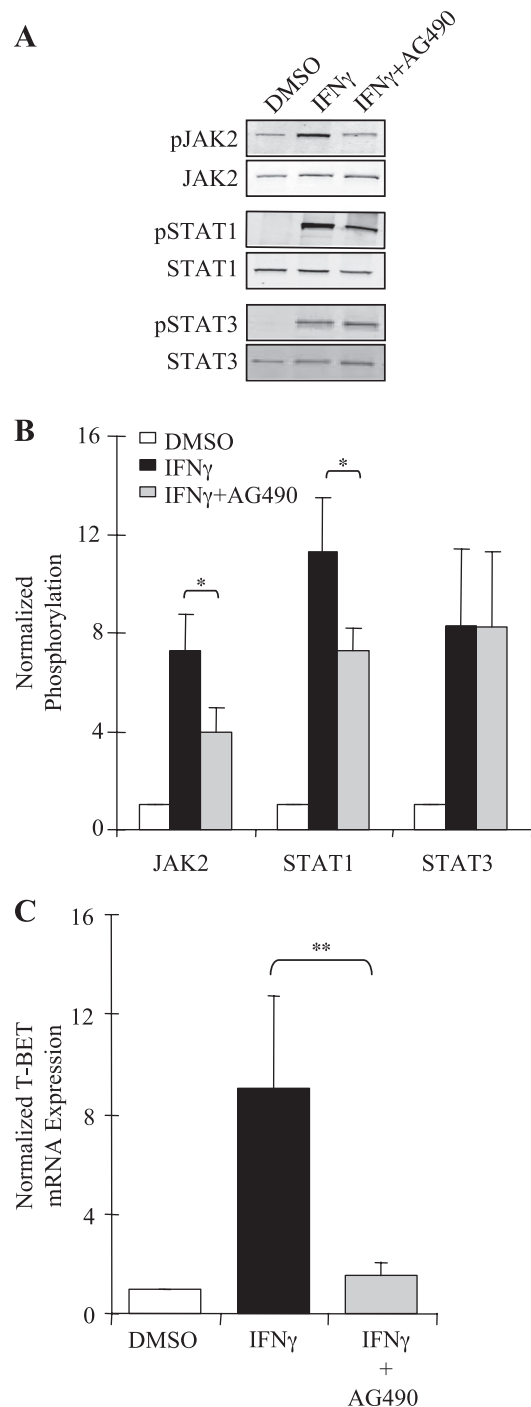


Fig. 2. T-bet expression in human ASMC is mediated by JAK/STAT signaling. Human ASMC cultures were serum starved for 24 h before a 15-min treatment with 10 ng/ml IFN γ in the presence of the 0.1% DMSO vehicle or 50 μ M AG-490. A: representative immunoblots are shown for phosphorylated and nonphosphorylated forms of JAK2, STAT1, and STAT3. B: Phosphorylation of JAK2, STAT1, and STAT3 was quantified by densitometry using phospho-specific antibodies and normalized to immunoreactivity from non-phospho-specific antibodies on the same membrane; $n = 3-6 \pm$ SE. C: total RNA was prepared from cultures treated for 24 h with 10 ng/ml IFN γ in the presence of DMSO or 50 μ M AG-490. T-bet expression was evaluated using TaqMan gene expression assays and normalized to 18S rRNA in the same samples; $n = 4 \pm$ SE. * $P < 0.05$, ** $P < 0.001$, significant difference.

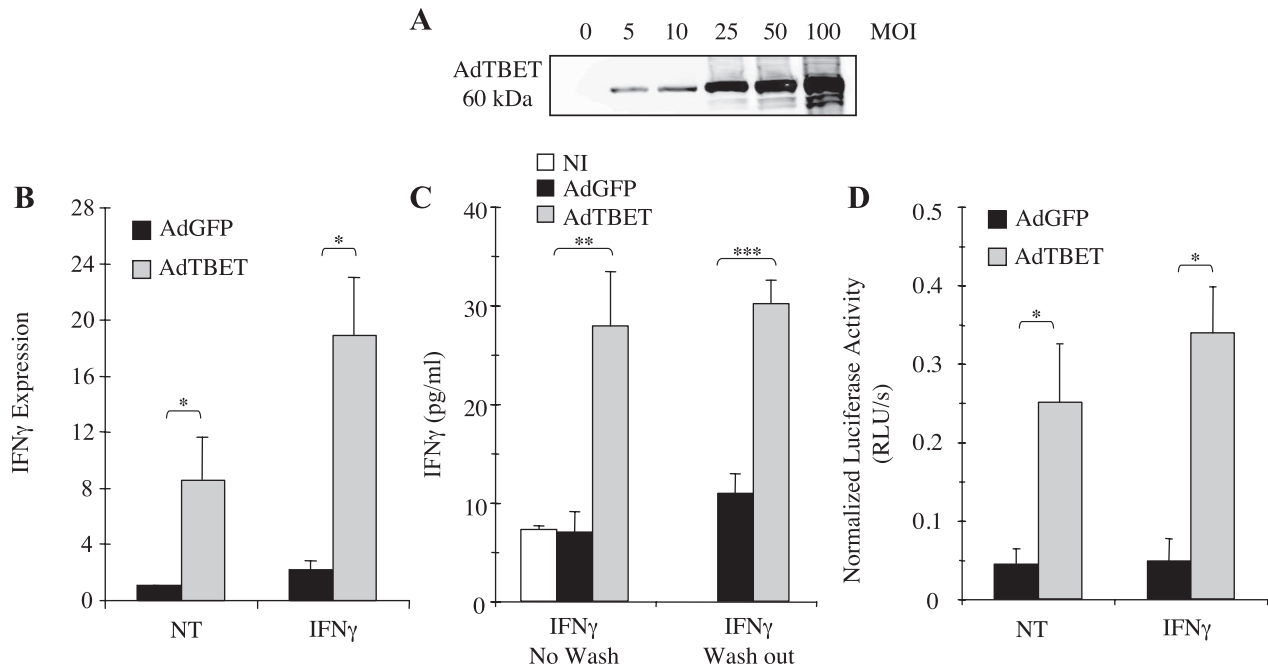


Fig. 3. T-bet expression stimulates IFN γ production in human ASMC. **A**: human ASMC were transduced with AdTBET at increasing MOI for 3 days and expression of T-bet was monitored by immunoblotting. In subsequent experiments, human ASMC were transduced with AdGFP or AdTBET at 20 MOI for 2 days followed by treatment with 10 ng/ml IFN γ for 48 h. **B**: IFN γ expression analyzed using TaqMan gene expression assays, normalized to 18S rRNA; $n = 3-4 \pm$ SE. **C**: IFN γ secretion was measured by ELISA in media from cells treated with 10 ng/ml IFN γ for 48 h without additional washing (no wash) or in IFN γ -treated cells washed with PBS following 24 h of treatment (wash out) and assayed 24 h later; $n = 3-4 \pm$ SE. NI, not infected. **D**: cultures were transduced with AdGFP or AdTBET at 20 MOI for 24 h followed by transfection with 5 μ g pGL3-mIFNG containing the mouse *IFNG* promoter and treatment with 10 ng/ml IFN γ for 2 h. Firefly luciferase activity was normalized to *Renilla* luciferase activity from cotransfected pRL; $n = 4-6 \pm$ SE. * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$, significant difference.

cantly increased IFN γ secretion to 27.9 pg/ml. This increase in IFN γ is attributed to the expression of AdTBET and not adenovirus infection itself since levels of IFN γ secretion were essentially the same in both noninfected and AdGFP cells. We distinguished between exogenous IFN γ and secreted IFN γ by conducting a pulse-chase experiment whereby ASMC cultures were treated with IFN γ for 24 h, followed by washing in PBS to remove exogenous IFN γ and addition of fresh media for analysis 24 h later. The concentration of IFN γ in these wash out experiments is similar to that seen with 48 h of IFN γ exposure with 10.9 pg/ml detected in AdGFP cells and 30.1 pg/ml in AdTBET cells, leading to the conclusion that we are measuring secreted IFN γ and not exogenous IFN γ . Thus expression of T-bet in human ASMC stimulates production of the Th1 cytokine IFN γ . The effect of T-bet on IFN γ -stimulated IFN γ expression was further evaluated by examining activity at the IFN γ promoter (Fig. 3D). No change in promoter activity was noted in AdGFP cells. In contrast, expression of AdTBET stimulated basal promoter activity 5.6-fold, which was further stimulated, although not significantly, by addition of IFN γ . This indicates that T-bet expression alone can modulate IFN γ promoter activity in the absence of stimulus, which correlates with previous observations in T cells (52). To further address the role of T-bet in IFN γ -stimulated IFN γ secretion, we knocked down AdTBET with T-bet specific siRNA. Of the three siRNA tested, the siRNA targeting exon 3 (siRNA 2) knocked down AdTBET expression by $\sim 50\%$ (Fig. 4A). This resulted in a decrease in IFN γ secretion of 16.9 from 26.8 pg/ml in cells transfected with the negative siRNA control compared with 30.11 pg/ml in nontreated cultures. This sup-

ports data demonstrating that expression of T-bet induces IFN γ expression and secretion in human ASMC.

T-bet expression affects cytokine and chemokine expression in human ASMC. We further analyzed effects of T-bet on secretion of inflammatory mediators in human ASMC using Luminex multibead assays specific for Th1/Th2 cytokines and chemokines. In noninfected or AdGFP-infected cells, we did not detect IL-1 α/β , IL-2, IFN $\alpha 2$, IL-12, IL-4, IL-5, IL-10, or IL-13 at basal levels or upon stimulation of IFN γ (data not shown). Treatment with IFN γ in noninfected ASMC stimulated secretion of monocyte chemoattractant protein (MCP)-1/CCL2 and eotaxin/CCL11 and induced secretion of regulated on activation normal T-expressed and secreted (RANTES)/CCL5 and TNF α (Table 1), and infection with AdGFP did not produce any significant differences in chemokine secretion (Fig. 5A). This again demonstrates that adenovirus infection alone does not affect secretion of the mediators analyzed. Upon expression of AdTBET, IFN γ -stimulated secretion of MCP-1/CCL2, RANTES/CCL5, and eotaxin/CCL11 was significantly reduced. Previous reports (37) describing the effects of T-bet overexpression on cytokine and chemokine receptor expression led to examination of the IL-12 receptor subunit IL-12R $\beta 2$ and the eotaxin receptor CCR3 (Fig. 5B). IL-12R $\beta 2$ expression was stimulated 2.6-fold in AdTBET cells, but further increases in IFN γ -stimulated IL-12R $\beta 2$ expression were blocked. In contrast, expression of AdTBET significantly decreases CCR3 expression and IFN γ fails to further modulate CCR3 in AdGFP or AdTBET cultures. We also examined the effect of IFN γ on TNF α since synergistic interactions between these mediators have been reported in

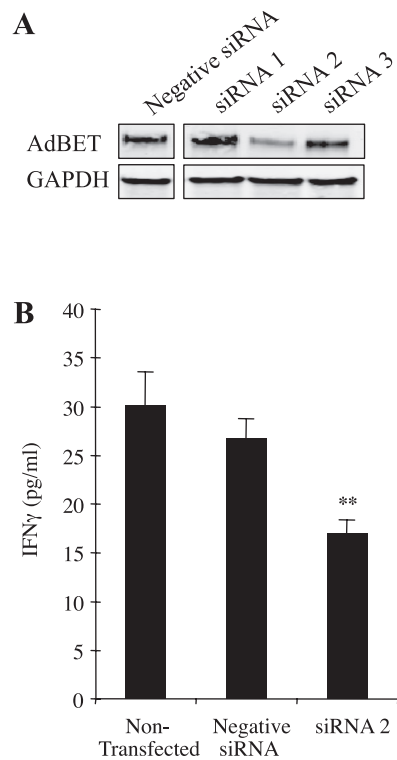


Fig. 4. Small-interfering (si)RNA-mediated knockdown of T-bet expression inhibits IFN γ secretion. Human ASM cells were transfected with AdTBET at 20 MOI for 24 h before transfection with 30 nM siRNA targeting T-bet. **A**: representative immunoblot demonstrating knockdown of T-bet expression 72 h after transfection. **B**: IFN γ secretion measured by ELISA in cultures transfected with the siRNA targeting exon 3 and treated with 10 ng/ml IFN γ for 48 h; $n = 6 \pm$ SE. $**P < 0.001$, significant difference.

ASMC (9, 54). AdTBET produces a modest but significant 1.78-fold increase in TNF α expression that is increased, although not significantly, by further IFN γ stimulation (Fig. 5B) and correlates with an increase in TNF α secretion in these cultures (Fig. 5A). These data correlate with previous studies in polarized Th2 cells where expression of T-bet increases TNF α secretion, accompanied by concomitant downregulation of Th2 cytokines and chemokine receptors (37).

T-bet reduces chemotactic migration in ASMC. Smooth muscle cell migration has been proposed to be an early event affecting airway remodeling that contributes to hyperplasia (17), and many of the growth factors and cytokines synthesized by ASMC are promigratory (22, 41), while β -adrenergic agonists

Table 1. Effect of IFN γ on chemokine secretion

	No Treatment	IFN γ
MCP-1/CCL2	1,941 $\pm$ 665	3,730 $\pm$ 1010
RANTES/CCL5	N.D.	885 $\pm$ 4*
Eotaxin/CCL11	465 $\pm$ 213	3,138 $\pm$ 1861
TNF α	N.D.	2,435 $\pm$ 105*
TNF β	N.D.	N.D.

Values are means $\pm$ SE. Data are reported in pg/ml from 3 donors analyzed in triplicate. Culture media from human airway smooth muscle cells were collected after 48 h of treatment with 10 ng/ml IFN γ and 50 μ l analyzed on a Luminex 100 using the Beadlyte human multicytokine detection system. MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation normal T-expressed and presumably secreted; N.D., not detected. $*P < 0.0001$, significant difference.

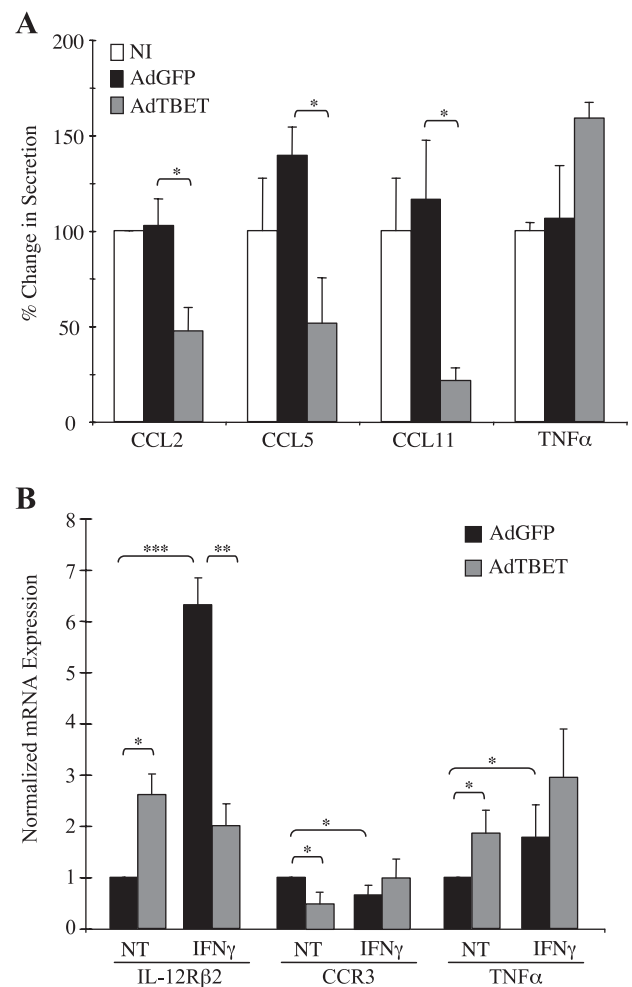


Fig. 5. T-bet expression affects cytokine and chemokine expression in human ASMC. Human ASMC were transfected with AdGFP or AdTBET at 20 MOI for 2 days followed by treatment with 10 ng/ml IFN γ for 48 h. **A**: media were collected for Luminex assays and results expressed as the % change in secretion from noninfected (NI) cultures treated with IFN γ . **B**: RNA was isolated for qPCR analysis, normalized to 18S rRNA; $n = 3-5 \pm$ SE. $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$, significant difference.

and corticosteroids commonly used to treat asthma inhibit migration (18). ASMC isolated from asthmatics also exhibit increased expression of CCR3 with induction of ASMC migration in response to eotaxin/CCL11 (27). The reduction of CCL11 secretion and CCR3 expression with T-bet expression led us to further examine whether T-bet altered migration of human ASMC (Fig. 6). In response to 1% serum or 10 ng/ml PDGF as chemoattractants, significant migration of AdGFP-infected human ASMC was noted. In ASMC expressing AdTBET, migration was dramatically reduced, with 43% less migration seen in response to serum and 56% less migration measured in response to PDGF. These results demonstrate that in contrast to reports in T cells (37), T-bet acts in human ASMC to reduce chemotactic migration and decreases in eotaxin secretion or activity through its receptor may be one mechanism mediating this response.

DISCUSSION

The studies described here have identified expression and activity of T-bet in human ASMC, a key structural cell type in

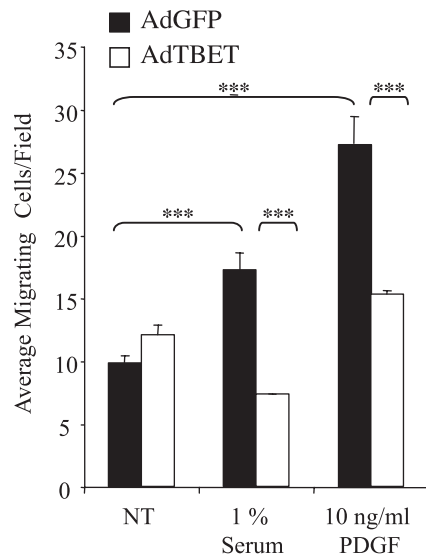


Fig. 6. T-bet reduces chemotactic migration of human ASM. ASM cultures were transduced with AdGFP or AdTBET at 20 MOI for 2 days, trypsinized, and plated for migration assays at a density of 5.0×10^4 per well. Migration proceeded for 5 h using 1% serum or 10 ng/ml PDGF as chemoattractants. Migrating cells were fixed and counted as described. Results are representative of 3 separate experiments performed in duplicate with 7 fields counted per treatment $\pm$ SE. *** $P < 0.0001$, significant difference.

the lung. The notion that T-bet may be expressed in ASM evolved from data generated in T-bet knockout mice, which exhibit airway hyperreactivity and smooth muscle remodeling characteristic of asthma in humans (15). T-bet expression was not identified in smooth muscle in these studies. However, ASM in these mice have increased spontaneous intracellular Ca^{2+} concentrations and higher acetylcholine-induced Ca^{2+} transients that contribute to increased basal airway tone and AHR (6). Data presented here demonstrate that smooth cell expression and activity of T-bet can be induced in ASM in vitro by an $\text{IFN}\gamma$ -stimulated Th1 response. Moreover, the JAK/STAT signaling pathways regulating T-bet in immune cells are intact in human ASM and control T-bet expression through upstream activation of JAK2/STAT1 signaling by $\text{IFN}\gamma$. A growing body of evidence demonstrates that T cells and ASM can interact via cell adhesion molecules (38) and ASM express functional antigen presenting proteins such as CD40, IgG receptors including $\text{Fc}\gamma\text{RII}$ and the pattern-recognition Toll-like receptors (10). Expression of T-bet further indicates that ASM are an important immunomodulatory cell type that utilizes transcriptional machinery found in T cells to respond to the inflammatory environment in the lung.

Analysis of target genes affected by T-bet in T cells demonstrates that in addition to $\text{IFN}\gamma$, T-bet drives expression of IL-2, IL-12R β 2, CCR3, and $\text{TNF}\alpha$ while reducing expression of IL-4, IL-5, and IL-13 (37). In human ASM, we found that in the absence of $\text{IFN}\gamma$, T-bet did stimulate expression of IL-12R β 2 and $\text{TNF}\alpha$ but reduced levels of CCR3 along with eotaxin/CCL11 secretion. It is clear from the results reported here that, while ASM retain many of the regulatory and functional effects of T-bet, ASM do not entirely recapitulate the entire T cell response to T-bet expression. This may be attributed to the absence of additional signals in ASM from Th1/Th2 cytokines including IL-2, IL-4, IL-5, or IL-13, which

were not detected in $\text{IFN}\gamma$ -stimulated or T-bet expressing ASM. This result is not entirely surprising since although ASM can respond to these cytokines in numerous ways (25), only IL-5 expression has been reported in ASM exposed to atopic asthmatic serum (21).

With induction of T-bet, we also observed concomitant downregulation of GATA-3 expression, which to our knowledge has not been studied in smooth muscle. In Th2 cells, GATA-3 directs expression of Th2 cytokines while repressing Th1 cytokine production (58). Retroviral transfer of GATA-3 into Th1 cells reduces $\text{IFN}\gamma$ production and increases IL-4 and IL-5 (12). The expression of GATA-3 is controlled by IL-4 activation of STAT6, and STAT6-deficient mice sensitized to ovalbumin do not develop eosinophilia and produce little airway inflammation following allergen challenge (2). Intranasal administration of antisense GATA-3 also reduces airway inflammation, hyperreactivity, and mucus production (13). This suggests that inhibition of Th2 cytokine production due to absence of GATA-3 activity will alleviate hallmark features of asthma and, in fact, GATA-3 expression is increased in asthmatic patients (42). GATA-3 may thus be an overlooked target of Th1/Th2 cytokine synthesis in human ASM whose functions will be the basis of future work. Additionally, an analysis of target genes affected by T-bet and GATA-3 demonstrates that these opposing transcription factors share many target genes when expressed in the same cell (26).

The role of $\text{IFN}\gamma$ in the pathogenesis asthma is complex and may depend on disease severity, route of administration, and duration of treatment, as well as interactions with other inflammatory mediators [reviewed by Tliba and Amrani (53)]. Reports of reduced $\text{IFN}\gamma$ production in T cells of asthmatics (30, 39) are in contrast to others that find increased $\text{IFN}\gamma$ levels in asthmatic BAL fluid (7) and sputum (8). In experimental models of asthma, exogenous administration of $\text{IFN}\gamma$ can suppress many aspects of allergic inflammation, including immunoglobulin E production, eosinophilia, and AHR, with differences seen between nebulized or parenteral $\text{IFN}\gamma$ administration (24, 35). However, in $\text{IFN}\gamma$ -deficient mice, the protective effects of $\text{IFN}\gamma$ on AHR after 1 wk of treatment were not observed with prolonged treatment for 4 wk (57). Additionally, in mouse models of chronic asthma, $\text{IFN}\gamma$ production is actually increased and treatment with neutralizing $\text{IFN}\gamma$ antibodies suppresses AHR with little effect on inflammation (33, 34). These conflicting functions of $\text{IFN}\gamma$ may be attributed to cross talk with other inflammatory cytokines such as IL-13 and $\text{TNF}\alpha$. When administered separately, $\text{IFN}\gamma$ inhibits IL-13-induced hyperplasia and eosinophilia, but coadministration with IL-13 potentiates effects of IL-13 on inflammation (16). Many other studies have demonstrated that when used in combination, $\text{IFN}\gamma$ and $\text{TNF}\alpha$ synergistically activate STAT- and NF- κ B-mediated transcription (43), which may be explained by upregulation of their respective receptors (32, 49). In our study, expression of T-bet increased $\text{IFN}\gamma$ -stimulated $\text{TNF}\alpha$ expression, and further studies will examine whether these chemokines interact to further modulate T-bet functions in ASM. The importance, however, of interferons to ASM functions should not be overlooked. Both type I ($\text{IFN}\alpha$ and $\text{IFN}\beta$) and type II ($\text{IFN}\gamma$) interferons inhibit proliferation of ASM (3, 55), while stimulating protein synthesis through mammalian target of rapamycin (mTOR)/S6K1 signaling (19). $\text{IFN}\beta$ and $\text{IFN}\gamma$ in the same study also increased contractile

protein expression without affecting cell size, suggesting that interferons may contribute to ASM remodeling in asthma. In our study, we were able to demonstrate that, in addition to IFN γ , IFN α could also stimulate T-bet expression in ASMC. This supports a role for both type I and type II IFNs in the inflammatory response of ASM.

Our understanding of the role of ASM in the lung has been primarily based on in vitro studies such as those presented here. Thus, while the identification and regulation of T-bet expression in human ASMC supports the concept that structural cells of the lung are important components of the immune response, further in vivo investigations are necessary to establish the role of ASM in this response. This is underscored by a recent perspective on the role of ASM in lung disease that highlights the need to identify and determine the physiologic relevance of inflammatory mediators expressed in ASM in vivo and whether these ASM-derived mediators can be effective therapeutic targets (44). Thus identification of T-bet and a characterization its functions in ASMC in vitro are the first steps in determining the importance of T-bet expression to ASM functions in the lung.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

- Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, Murphy TL, Murphy KM. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4 $^{+}$ T cells. *Nat Immunol* 3: 549–557, 2002.
- Akimoto T, Numata F, Tamura M, Takata Y, Higashida N, Takashi T, Takeda K, Akira S. Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J Exp Med* 187: 1537–1542, 1998.
- Amrani Y, Tliba O, Choubey D, Huang CD, Krymskaya VP, Eszterhas A, Lazaar AL, Panettieri RA Jr. IFN-gamma inhibits human airway smooth muscle cell proliferation by modulating the E2F-1/Rb pathway. *Am J Physiol Lung Cell Mol Physiol* 284: L1063–L1071, 2003.
- Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 19: 2499, 1991.
- Bentley AM, Hamid Q, Robinson DS, Schotman E, Meng Q, Assoufi B, Kay AB, Durham SR. Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa. *Am J Respir Crit Care Med* 153: 551–556, 1996.
- Bergner A, Kellner J, Silva AK, Gamarra F, Huber RM. Ca $^{2+}$ -signaling in airway smooth muscle cells is altered in T-bet knock-out mice. *Respir Res* 7: 33, 2006.
- Boniface S, Koscher V, Mamessier E, El Biaze M, Dupuy P, Lorec AM, Guillot C, Badier M, Bongrand P, Vervloet D, Magnan A. Assessment of T lymphocyte cytokine production in induced sputum from asthmatics: a flow cytometry study. *Clin Exp Allergy* 33: 1238–1243, 2003.
- Cho SH, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4 $^{+}$ and CD8 $^{+}$ T cells in atopic asthma. *Am J Respir Crit Care Med* 171: 224–230, 2005.
- Clarke DL, Clifford RL, Jindarat S, Proud D, Pang L, Belvisi M, Knox AJ. TNF α and IFN γ synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF-kappaB, and the transcriptional coactivator CREB-binding protein. *J Biol Chem* 285: 29101–29110, 2010.
- Damera G, Tliba O, Panettieri RA Jr. Airway smooth muscle as an immunomodulatory cell. *Pulm Pharmacol Ther* 22: 353–359, 2009.
- Dechert MA, Holder JM, Gerthoffer WT. p21-activated kinase 1 participates in tracheal smooth muscle cell migration by signaling to p38 MAPK. *Am J Physiol Cell Physiol* 281: C123–C132, 2001.
- Ferber IA, Lee HJ, Zonin F, Heath V, Mui A, Arai N, O'Garra A. GATA-3 significantly downregulates IFN-gamma production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. *Clin Immunol* 91: 134–144, 1999.
- Finotto S, De Sanctis GT, Lehr HA, Herz U, Buerke M, Schipp M, Bartsch B, Atreya R, Schmitt E, Galle PR, Renz H, Neurath MF. Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression. *J Exp Med* 193: 1247–1260, 2001.
- Finotto S, Glimcher L. T cell directives for transcriptional regulation in asthma. *Springer Semin Immunopathol* 25: 281–294, 2004.
- Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, Ackerman K, Haley K, Galle PR, Szabo SJ, Drazen JM, De Sanctis GT, Glimcher LH. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 295: 336–338, 2002.
- Ford JG, Rennick D, Donaldson DD, Venkayya R, McArthur C, Hansell E, Kurup VP, Warnock M, Grunig G. IL-13 and IFN-gamma: interactions in lung inflammation. *J Immunol* 167: 1769–1777, 2001.
- Gerthoffer WT. Migration of airway smooth muscle cells. *Proc Am Thorac Soc* 5: 97–105, 2008.
- Goncharova EA, Billington CK, Irani C, Vorotnikov AV, Tkachuk VA, Penn RB, Krymskaya VP, Panettieri RA Jr. Cyclic AMP-mobilizing agents and glucocorticoids modulate human smooth muscle cell migration. *Am J Respir Cell Mol Biol* 29: 19–27, 2003.
- Goncharova EA, Lim PN, Chisolm A, Fogle HW, Taylor JH III, Goncharov DA, Eszterhas A, Panettieri RA Jr, Krymskaya VP. Interferons modulate mitogen-induced protein synthesis in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 299: L25–L35, 2010.
- Graham FL, Prevec L. Methods for construction of adenovirus vectors. *Mol Biotechnol* 3: 207–220, 1995.
- Hakonarson H, Maskeri N, Carter C, Grunstein MM. Regulation of TH1- and TH2-type cytokine expression and action in atopic asthmatic sensitized airway smooth muscle. *J Clin Invest* 103: 1077–1087, 1999.
- Hedges JC, Dechert MA, Yamboliev IA, Martin JL, Hickey E, Weber LA, Gerthoffer WT. A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem* 274: 24211–24219, 1999.
- Hedges JC, Singer CA, Gerthoffer WT. Mitogen-activated protein kinases regulate cytokine gene expression in human airway myocytes. *Am J Respir Cell Mol Biol* 23: 86–94, 2000.
- Hofstra CL, Van Ark I, Hofman G, Nijkamp FP, Jardieu PM, Van Oosterhout AJ. Differential effects of endogenous and exogenous interferon-gamma on immunoglobulin E, cellular infiltration, and airway responsiveness in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 19: 826–835, 1998.
- Howarth PH, Knox AJ, Amrani Y, Tliba O, Panettieri RA Jr, Johnson M. Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* 114: S32–50, 2004.
- Jenner RG, Townsend MJ, Jackson I, Sun K, Bouwman RD, Young RA, Glimcher LH, Lord GM. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. *Proc Natl Acad Sci USA* 106: 17876–17881, 2009.
- Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q, Lamkhioued B. CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* 175: 2702–2708, 2005.
- Kiwamoto T, Ishii Y, Morishima Y, Yoh K, Maeda A, Ishizaki K, Iizuka T, Hegab AE, Matsuno Y, Homma S, Nomura A, Sakamoto T, Takahashi S, Sekizawa K. Transcription factors T-bet and GATA-3 regulate development of airway remodeling. *Am J Respir Crit Care Med* 174: 142–151, 2006.

29. Ko FW, Lun SW, Wong CK, Szeto CC, Lam CW, Leung TF, Hui DS. Decreased T-bet expression and changes in chemokine levels in adults with asthma. *Clin Exp Immunol* 147: 526–532, 2007.
30. Koning H, Neijens HJ, Baert MR, Oranje AP, Savelkoul HF. T cell subsets and cytokines in allergic and non-allergic children. I. Analysis of IL-4, IFN-gamma and IL-13 mRNA expression and protein production. *Cytokine* 9: 416–426, 1997.
31. Koning H, Neijens HJ, Baert MR, Oranje AP, Savelkoul HF. T cells subsets and cytokines in allergic and non-allergic children. II. Analysis of IL-5 and IL-10 mRNA expression and protein production. *Cytokine* 9: 427–436, 1997.
32. Krakauer T, Oppenheim JJ. IL-1 and tumor necrosis factor-alpha each up-regulate both the expression of IFN-gamma receptors and enhance IFN-gamma-induced HLA-DR expression on human monocytes and a human monocytic cell line (THP-1). *J Immunol* 150: 1205–1211, 1993.
33. Kumar RK, Herbert C, Webb DC, Li L, Foster PS. Effects of anticytokine therapy in a mouse model of chronic asthma. *Am J Respir Crit Care Med* 170: 1043–1048, 2004.
34. Kumar RK, Webb DC, Herbert C, Foster PS. Interferon-gamma as a possible target in chronic asthma. *Inflamm Allergy Drug Targets* 5: 253–256, 2006.
35. Lack G, Renz H, Saloga J, Bradley KL, Loader J, Leung DY, Larsen G, Gelfand EW. Nebulized but not parenteral IFN-gamma decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J Immunol* 152: 2546–2554, 1994.
36. Lack G, Renz H, Saloga J, Bradley KL, Loader J, Leung DY, Larsen G, Gelfand EW. Nebulized but not parenteral IFN-gamma decreases IgE production and normalizes airways function in a murine model of allergen sensitization. *J Immunol* 152: 2546–2554, 1994.
37. Lametschwandtner G, Biedermann T, Schwarzler C, Gunther C, Kund J, Fassl S, Hinteregger S, Carballido-Perrig N, Szabo SJ, Glimcher LH, Carballido JM. Sustained T-bet expression confers polarized human TH2 cells with TH1-like cytokine production and migratory capacities. *J Allergy Clin Immunol* 113: 987–994, 2004.
38. Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* 180: 807–816, 1994.
39. Leonard C, Tormey V, Burke C, Poulter LW. Allergen-induced cytokine production in atopic disease and its relationship to disease severity. *Am J Respir Cell Mol Biol* 17: 368–375, 1997.
40. Leung DY, Martin RJ, Szefer SJ, Sher ER, Ying S, Kay AB, Hamid Q. Dysregulation of interleukin 4, interleukin 5, and interferon gamma gene expression in steroid-resistant asthma. *J Exp Med* 181: 33–40, 1995.
41. Madison JM. Migration of airway smooth muscle cells. *Am J Respir Cell Mol Biol* 29: 8–11, 2003.
42. Nakamura Y, Ghaffar O, Olivenstein R, Taha RA, Soussi-Gounni A, Zhang DH, Ray A, Hamid Q. Gene expression of the GATA-3 transcription factor is increased in atopic asthma. *J Allergy Clin Immunol* 103: 215–222, 1999.
43. Ohmori Y, Schreiber RD, Hamilton TA. Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J Biol Chem* 272: 14899–14907, 1997.
44. Panettieri RA Jr, Kotlikoff MI, Gerthoffer WT, Hershenson MB, Woodruff PG, Hall IP, Banks-Schlegel S. Airway smooth muscle in bronchial tone, inflammation, and remodeling: basic knowledge to clinical relevance. *Am J Respir Crit Care Med* 177: 248–252, 2008.
45. Panettieri RA Jr, Lazaar AL, Pure E, Albelda SM. Activation of cAMP-dependent pathways in human airway smooth muscle cells inhibits TNF-alpha-induced ICAM-1 and VCAM-1 expression and T lymphocyte adhesion. *J Immunol* 154: 2358–2365, 1995.
46. Pene J, Rousset F, Briere F, Chretien I, Bonnefoy JY, Spits H, Yokota T, Arai N, Arai K, Banchereau J. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E2. *Proc Natl Acad Sci USA* 85: 6880–6884, 1988.
47. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 326: 298–304, 1992.
48. Robinson DS, Lloyd CM. Asthma: T-bet—a master controller? *Curr Biol* 12: R322–R324, 2002.
49. Ruggiero V, Tavernier J, Fiers W, Baglioni C. Induction of the synthesis of tumor necrosis factor receptors by interferon-gamma. *J Immunol* 136: 2445–2450, 1986.
50. Simon AR, Takahashi S, Severgnini M, Fanburg BL, Cochran BH. Role of the JAK-STAT pathway in PDGF-stimulated proliferation of human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 282: L1296–L1304, 2002.
51. Singer CA, Baker KJ, McCaffrey A, AuCoin DP, Dechert MA, Gerthoffer WT. p38 MAPK and NF-kappaB mediate COX-2 expression in human airway myocytes. *Am J Physiol Lung Cell Mol Physiol* 285: L1087–L1098, 2003.
52. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669, 2000.
53. Tliba O, Amrani Y. Airway smooth muscle cell as an inflammatory cell: lessons learned from interferon signaling pathways. *Proc Am Thorac Soc* 5: 106–112, 2008.
54. Tliba O, Cidlowski JA, Amrani Y. CD38 expression is insensitive to steroid action in cells treated with tumor necrosis factor-alpha and interferon-gamma by a mechanism involving the up-regulation of the glucocorticoid receptor beta isoform. *Mol Pharmacol* 69: 588–596, 2006.
55. Tliba O, Tliba S, Da Huang C, Hoffman RK, DeLong P, Panettieri RA Jr, Amrani Y. Tumor necrosis factor alpha modulates airway smooth muscle function via the autocrine action of interferon beta. *J Biol Chem* 278: 50615–50623, 2003.
56. Ying S, Durham SR, Corrigan CJ, Hamid Q, Kay AB. Phenotype of cells expressing mRNA for TH2-type (interleukin 4 and interleukin 5) and TH1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am J Respir Cell Mol Biol* 12: 477–487, 1995.
57. Yoshida M, Leigh R, Matsumoto K, Wattie J, Ellis R, O'Byrne PM, Inman MD. Effect of interferon-gamma on allergic airway responses in interferon-gamma-deficient mice. *Am J Respir Crit Care Med* 166: 451–456, 2002.
58. Zhang DH, Cohn L, Ray P, Bottomly K, Ray A. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem* 272: 21597–21603, 1997.