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

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Submitted 12 May 2010; accepted in final form 11 January 2011

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 (Th1) 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 IFNγ. T-bet-deficient mice develop airway hyperreactivity, undergo remodeling, and exhibit defects in IFNγ 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 (ASM) by IFNγ. This IFNγ-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 IFNγ-stimulated IFNγ expression, secretion, and promoter activity, while inhibiting IFNγ-stimulated release of chemokines including monocyte chemotactic 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 IL12Rβ2 and Tnfrs. T-bet expression also reduces chemotactic migration of ASM 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 (Th1)/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 IFNγ, and the cells continue to synthesize IL-2, IL-12, and IFNγ. 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 IFNγ 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 IFNγ (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 IFNγ (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 IFNγ production. Expression of T-bet into Th2 cells directs these cells to a Th1 phenotype by inducing IFNγ synthesis, while repressing IL-4 and IL-5 production (52) and increasing expression of chemokine receptors including IL-12Rβ2 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 (ASM) 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 ASM. We present data demonstrating that the expression and activity of T-bet are induced by IFNγ in human ASM. We further examine functions of T-bet on cytokine and chemokine secretion and receptor expression, as well as chemotactic migration of human ASM. 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 ASM may provide insights into new therapeutic targets inflammatory airway disease.

METHODS

Smooth muscle cell culture and treatments. Normal ASM 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
M199 media supplemented with 5% normal calf serum (NCS), 0.5 ng/ml epidermal growth factor, 5 μg/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. IFNγ (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 μM; Calbiochem, San Diego, CA) for 15 min before and during IFNγ stimulation. For adenosival transduction, confluent cultures were infected in 200 μl 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-1β, TNFα, and IFNγ for 20 h. Total RNA was extracted with TRIzol reagent (Invitrogen) and treated with DNase I (1U/μl) at 37°C for 15 min. RNA concentrations were quantified by measuring absorbance at 260 nm. First-strand cDNA synthesis was performed from 2 μg total RNA using SuperScript II reverse transcriptase. T-bet specific oligonucleotides were synthesized by Integrated DNA Technologies (Corvalle, IA) from the following sequence based on NM_013351: 5′-CGACGGCTACGGGAAGGTG-3′ and 5′-TGT- CATCCTGCATCGTTGGAAAT-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 SalI 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 for quantitative evaluation T-bet expression in ASMC: 5′-ACTGGAGGTGCTCGGAAAATCTG-3′ and 5′-GCCGGGCTGGTACTTAT-GAG-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 (NH4)2SO4; 1.5 mM MgCl2; 0.25 mM dATP, dCTP, dGTP, dTTP; 10% DMSO; 50 μM of each primer; 5 μl 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 μg 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; IFNγ (IFNG), Hs00174145; IL2RB2, Hs0055486; CCL11, Hs00257013; and TNEF, 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 μM 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 (Jacon 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 μg total protein was normalized to that from nonphosphoprotein antibodies on the same membrane. For T-bet immunoblot, 50 μg 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.

EMSAs. 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 μmol double stranded oligonucleotides (Santa Cruz Biotechnologies, Santa Cruz, CA) end labeled with [γ-32P]ATP and 4 μg nuclear extract. Reactions were incubated at room temperature for 20 min and stopped with an appropriate volume of 10× 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 × 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 CaPO4 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 μg of pGL3 firefly luciferase DNA and 100 ng of pRL Renilla luciferase DNA to 2× N/A-nis (2-hydroxyl)-2-aminoethane-sulfonic acid buffered saline, pH 6.95, and 0.125 M CaCl2. Cultures were maintained for 4 h in a humidified 3% CO2 atmosphere at 37°C to increase transfection efficiency. After being washed thoroughly in PBS, cultures were returned to a 5% CO2 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 lumimeter (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 with the following sequences: Exon 6 (siRNA 1), 5′GGAGCUUUUUGACAUCGUU-3′; exon 3 (siRNA 2), 5′-GCCGAUGAUUUGACUCUATT-3′; and exon 5 (siRNA 3), 5′-GGUAUCGGGAGAACUUGTT-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. IFNγ 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-μm 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 DiffQuik Wright-Giemsa solution. The number of cells on the lower face of the filter was counted in seven fields under 10× 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 IFNγ in human ASM. To begin to elucidate functional consequences of T-bet expression in ASM, we examined T-bet expression in human ASM. Figure 1A, left, shows that while T-bet mRNA is not detected at basal levels in human ASM, treatment with 10 ng/ml IFNγ 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 IFNα, was also capable of up-regulating T-bet expression in human ASM (Fig. 1A, right). The time course of induction of T-bet was further investigated using qPCR (Fig. 1C). Treatment with IFNγ 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 IFNγ exposure. This increase in T-bet expression is seen with up to 48 h of IFNγ stimulation (data not shown). The biphasic increase in T-bet expression is likely due to a positive feedback loop by which IFNγ-stimulation of T-bet expression further stimulates IFNγ 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.

IFNγ-stimulation of T-bet-dependent DNA binding in human ASM. To determine whether induction of T-bet expression in human ASM 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 IFNγ for 2 h increased nuclear protein binding of the T-bet oligonucleo-
T-bet by IFN

regation of various STAT proteins. In T cells, the induction of the membrane-associated JAK and subsequent phosphorylation of human ASMC. Interferons transduce signals, in part, through results in T-bet-dependent DNA binding.

experiments demonstrate that increased expression of T-bet results in a mobility supershift (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 ASM. 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 IFNγ 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 ASM was examined by phospho-immunoblot analysis. In initial experiments, IFNγ 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 μM AG-490, a JAK2 inhibitor, significantly reduced IFNγ-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 ASM (50) with no apparent effects on JAK1. Treatment with AG-490 also blocks induction of T-bet expression by IFNγ, as determined by qPCR analysis (Fig. 2C). The results demonstrate that IFNγ does stimulate upstream JAK2/STAT1 signaling pathways, as recently described (9), and that JAK2/STAT1 activity mediates T-bet expression in human ASM.

T-bet expression stimulates IFNγ production. To facilitate the study of T-bet, a cDNA clone was isolated from human ASM 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 ASM were analyzed by examining the effect of AdTBET on IFNγ mRNA expression by qPCR (Fig. 3B). In cultures infected with the control AdGFP virus, IFNγ stimulation produced a 2.1-fold increase in IFNγ mRNA expression. Expression of AdTBET stimulated both basal and IFNγ-stimulated IFNγ mRNA expression by 8.6- and 18.9-fold, respectively. To correlate these changes in IFNγ expression with IFNγ secretion, we measured IFNγ by ELISA (Fig. 3C). In nontreated cells, IFNγ secretion was not detected. However, IFNγ 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 IFNγ secretion, while expression of AdTBET signifi-

Fig. 2. T-bet expression in human ASM is mediated by JAK/STAT signaling. Human ASM cultures were serum starved for 24 h before a 15-min treatment with 10 ng/ml IFNγ in the presence of the 0.1% DMSO vehicle or 50 μM 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 ± SE. C: total RNA was prepared from cultures treated for 24 h with 10 ng/ml IFNγ in the presence of DMSO or 50 μM AG-490. T-bet expression was evaluated using TaqMan gene expression assays and normalized to 18S rRNA in the same samples; n = 4 ± SE. *P < 0.05, **P < 0.001, significant difference.
cantly increased IFNγ secretion to 27.9 pg/ml. This increase in IFNγ is attributed to the expression of AdTBET and not adenovirus infection itself since levels of IFNγ secretion were essentially the same in both noninfected and AdGFP cells. We distinguished between exogenous IFNγ and secreted IFNγ by conducting a pulse-chase experiment whereby ASMC cultures were treated with IFNγ for 24 h, followed by washing in PBS to remove exogenous IFNγ and addition of fresh media for analysis 24 h later. The concentration of IFNγ in these wash out experiments is similar to that seen with 48 h of IFNγ 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 IFNγ and not exogenous IFNγ. Thus expression of T-bet in human ASMC stimulates production of the Th1 cytokine IFNγ. The effect of T-bet on IFNγ-stimulated IFNγ expression was further evaluated by examining activity at the IFNγ 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 IFNγ. This indicates that T-bet expression alone can modulate IFNγ 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 IFNγ-stimulated IFNγ 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 ~50% (Fig. 4A). This resulted in a decrease in IFNγ 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 supports data demonstrating that expression of T-bet induces IFNγ 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-1α/β, IL-2, IFNα2, IL-12, IL-4, IL-5, IL-10, or IL-13 at basal levels or upon stimulation of IFNγ (data not shown). Treatment with IFNγ 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 TNFα (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, IFNγ-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-12Rβ2 and the eotaxin receptor CCR3 (Fig. 5B). IL-12Rβ2 expression was stimulated 2.6-fold in AdTBET cells, but further increases in IFNγ-stimulated IL-12Rβ2 expression were blocked. In contrast, expression of AdTBET significantly decreases CCR3 expression and IFNγ fails to further modulate CCR3 in AdGFP or AdTBET cultures. We also examined the effect of IFNγ on TNFα since synergistic interactions between these mediators have been reported in
ASMC (9, 54). AdTBET produces a modest but significant 1.78-fold increase in TNFα/H9251 expression that is increased, although not significantly, by further IFNγ/H9253 stimulation (Fig. 5B) and correlates with an increase in TNFα/H9251 secretion in these cultures (Fig. 5A). These data correlate with previous studies in polarized Th2 cells where expression of T-bet increases TNFα/H9251 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 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 the airway. The downregulation of CCL11 and CCR3 expression in cells expressing T-bet suggests a possible therapeutic strategy for asthma, as these chemokines play a significant role in airway remodeling and eosinophil recruitment. The reduction in eotaxin expression and CCR3 correlates with the decrease in chemotactic migration seen in human ASMC infected with AdTBET.

### Table 1. Effect of IFNγ on chemokine secretion

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>No Treatment</th>
<th>IFNγ</th>
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<tr>
<td>MCP-1/CCL2</td>
<td>1,941 ± 665</td>
<td>3,730 ± 1010</td>
</tr>
<tr>
<td>RANTES/CCL5</td>
<td>N.D.</td>
<td>885 ± 4*</td>
</tr>
<tr>
<td>Eotaxin/CCL11</td>
<td>465 ± 213</td>
<td>3,138 ± 1861</td>
</tr>
<tr>
<td>TNFα</td>
<td>N.D.</td>
<td>2,435 ± 105*</td>
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<td>TNFβ</td>
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Values are means ± 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 IFNγ and 50 μl analyzed on a Luminex 100 using the Beadlyte human multicytokine detection system. MCP-1, monocyte chemotactrant protein-1; RANTES, regulated on activation normal T-expressed and presumably secreted; N.D, not detected. *P < 0.0001, significant difference.

Fig. 4. Small-interfering (si)RNA-mediated knockdown of T-bet expression inhibits IFNγ secretion. Human ASMC were transduced 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: IFNγ secretion measured by ELISA in cultures transfected with the siRNA targeting exon 3 and treated with 10 ng/ml IFNγ for 48 h. n = 6 ± SE. **P < 0.001, significant difference.

Fig. 5. T-bet expression affects cytokine and chemokine expression in human ASMC. Human ASMC were transduced with AdGFP or AdTBET at 20 MOI for 2 days followed by treatment with 10 ng/ml IFNγ for 48 h. A: media were collected for Luminex assays and results expressed as the % change in secretion from noninfected (NI) cultures treated with IFNγ. B: RNA was isolated for qPCR analysis, normalized to 18S rRNA; n = 3–5 ± SE. *P < 0.05; **P < 0.001; ***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, ASMC in these mice have increased spontaneous intracellular Ca²⁺ concentrations and higher acetylcholine-induced Ca²⁺ 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 ASMC in vitro by an IFNγ-stimulated Th1 response. Moreover, the JAK/STAT signaling pathways regulating T-bet in immune cells are intact in human ASMC and control T-bet expression through upstream activation of JAK2/STAT1 signaling by IFNγ. A growing body of evidence demonstrates that T cells and ASMC can interact via cell adhesion molecules (38) and ASMC express functional antigen presenting proteins such as CD40, IgG receptors including FcγRII and the pattern-recognition Toll-like receptors (10). Expression of T-bet further indicates that ASMC 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 IFNγ, T-bet drives expression of IL-2, IL-12Rβ2, CCR3, and TNFα while reducing expression of IL-4, IL-5, and IL-13 (37). In human ASMC, we found that in the absence of IFNγ, T-bet did stimulate expression of IL-12Rβ2 and TNFα but reduced levels of CCR3 along with eotaxin/CCL11 secretion. It is clear from the results reported here that, while ASMC retain many of the regulatory and functional effects of T-bet, ASMC do not entirely recapitulate the entire T cell response to T-bet expression. This may be attributed to the absence of additional signals in ASMC from Th1/Th2 cytokines including IL-2, IL-4, IL-5, or IL-13, which were not detected in IFNγ-stimulated or T-bet expressing ASMC. This result is not entirely surprising since although ASMC can respond to these cytokines in numerous ways (25), only IL-5 expression has been reported in ASMC 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 IFNγ 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 ASMC whose function 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 IFNγ in the pathogenesis of 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 IFNγ production in T cells of asthmatics (30, 39) are in contrast to others that find increased IFNγ levels in asthmatic BAL fluid (7) and sputum (8). In experimental models of asthma, exogenous administration of IFNγ can suppress many aspects of allergic inflammation, including immunoglobulin E production, eosinophilia, and AHR, with differences seen between nebulized or parenteral IFNγ administration (24, 35). However, in IFNγ-deficient mice, the protective effects of IFNγ on AHR after 1 wk of treatment were not observed with prolonged treatment for 4 wk (57). Additionally, in mouse models of chronic asthma, IFNγ production is actually increased and treatment with neutralizing IFNγ antibodies suppresses AHR with little effect on inflammation (33, 34). These conflicting functions of IFNγ may be attributed to cross talk with other inflammatory cytokines such as IL-13 and TNFα. When administered separately, IFNγ 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, IFNγ and TNFα synergistically activate STAT- and NF-κB-mediated transcription (43), which may be explained by upregulation of their respective receptors (32, 49). In our study, expression of T-bet increased IFNγ-stimulated TNFα expression, and further studies will examine whether these chemokines interact to further modulate T-bet functions in ASMC. The importance, however, of interferons to ASMC functions should not be overlooked. Both type I (IFNox and IFNβ) and type II (IFNγ) interferons inhibit proliferation of ASMC (3, 55), while stimulating protein synthesis through mammalian target of rapamycin (mTOR)/S6K1 signaling (19). IFNβ and IFNγ in the same study also increased contractile

**Fig. 6. T-bet reduces chemotactic migration of human ASMC.** ASMC 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 × 10⁴ 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 ± SE. ***P < 0.0001, significant difference.
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 IFNγ, IFNα could also stimulate T-bet expression in ASM. 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 ASM 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 ASM in vitro are the first steps in determining the importance of T-bet expression to ASM functions in the lung.

ACKNOWLEDGMENTS

I thank Shanti Rawat for excellent technical assistance, Dr. M. J. Townsend for the IFNγ promoter construct and Drs. William T. Gerthoffer, Dorothy Hudig, and Andrew R. Kuhn for helpful advice and critique of this work.

GRANTS

C. A. Singer is supported by a Career Development Award HL-080960.

DISCLOSURES

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

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