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Inhibition of Tumor Necrosis Factor-α–Inducible Inflammatory Genes by Interferon-γ Is Associated with Altered Nuclear Factor-κB Transactivation and Enhanced Histone Deacetylase Activity

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ABSTRACT

Airway smooth muscle (ASM) cells can act as effector cells in the initiation and/or perpetuation of airway inflammation in asthma by producing various inflammatory chemokines or cytokines. Previous studies from our laboratory and others showed that the combination of tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) or endogenous IFNβ results in a synergistic induction of various pro-inflammatory genes, including CD38 and regulated upon activation normal T-cell expressed and secreted (RANTES), in ASM cells. In contrast to these studies, we found that IFNγ (1000 U/ml) markedly inhibited TNFα-induced expression of interleukin (IL)-6, IL-8, and eotaxin by 66.29 $\pm$ 3.33, 43.86 $\pm$ 7.11, and 63.25 $\pm$ 6.46%, respectively. These genes were also found to be NF-κB-dependent in that TNFα-induced expression of IL-6, IL-8, and eotaxin was dose-dependently inhibited by the selective IKKβ inhibitor 4-(2′-aminoethyl)amino-1,8-dimethylimidazo[1,2-*a*]quinoxaline (BMS-345541) (1–30 μM). Using a luciferase reporter construct containing κB sites, we found that IFNγ (10–1000 U/ml) inhibits NF-κB–dependent gene transcription in a dose-dependent manner. Moreover, IFNγ failed to affect TNFα-induced IκKβ phosphorylation or IκB degradation as well as nuclear NF-κB/DNA interaction. It is noteworthy that IFNγ decreases TNFα-induced histone acetyl transferase (HAT) and increases histone deacetylase (HDAC) activities. Finally, trichostatin A, an HDAC inhibitor, prevents IFNγ inhibitory action on TNFα-induced gene expression. Together, our data indicate that IFNγ is a potent inhibitor of specific TNFα-inducible inflammatory genes by acting on NF-κB transactivation via the modulation of HDAC function.

In recent years, there has been a veritable explosion of articles showing that tumor necrosis factor (TNFα) represents a new promising target for the treatment of chronic inflammatory disorders such as asthma. Several reports used either pharmacological inhibitors or neutralizing antibodies (etanercept) both in animal models (Renzetti et al., 1996; Kim et al., 2006) and asthmatic subjects (Berry et al., 2006) to demonstrate that TNFα signaling is an important component in the pathogenesis of asthma. Among cytokines, TNFα is one of the most potent activators of NF-κB, a ubiquitously expressed transcription factor that plays a leading role in the expression of a number of cellular genes involved in immune, inflammatory proapoptotic and antiapoptotic responses. In many cell types, NF-κB complex exists in the cytoplasm as an inactive form through association with inhibitory proteins called inhibitors of NF-κB (IκBs). Treatment of cells with TNFα promotes a rapid activation of IKKβ of the IκK complex, leading to IκB phosphorylation and IκB degradation, resulting in nuclear translocation of NF-κB and in transcriptional machinery activation (Baldwin, 1996). More recent studies showed that NF-κB–dependent gene expression needs corepressors and coactivators involved in modifying chromatin structure via histone acetyltransferase/histone deacetylase (HAT/HDAC) activities for full transcriptional

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ABBREVIATIONS: TNFα, tumor necrosis factor α; IκB, inhibitor of κB; IκK, Iκ kinase; IFN, interferon; NF-κB, nuclear factor κB; HAT, histone acetyltransferase; HDAC, histone deacetylase; IL, interleukin; ASM, airway smooth muscle; RANTES, regulated upon activation normal T-cell expressed and secreted; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; TSA, trichostatin A; PCR, polymerase chain reaction; SEAP, secreted alkaline phosphatase; r, recombinant; BMS 345541, 4-(2′-aminoethyl)amino-1,8-dimethylimidazo[1,2-*a*]quinoxaline.

activation (Rahman et al., 2004). Several strategies have convincingly demonstrated the therapeutic value of NF-κB inhibitors in asthma. Allergen-associated asthmatic reactions could be significantly reduced in 1) knockout mice deficient in NF-κB/Rel genes (Das et al., 2001), 2) transgenic mice selectively overexpressing NF-κB regulatory proteins in the lung epithelial cells (IKKβ) (Poynter et al., 2002), and 3) animals treated with the potential IKKβ inhibitor 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (Birrell et al., 2005). Thus, targeting TNFα signaling, including NF-κB pathways, represents a promising therapeutic option for the treatment of asthma.

Several lines of evidence demonstrate that TNFα could play a role in the pathogenesis of asthma through a direct immuno-modulatory action on ASM cells (Halayko and Amrani, 2003). In cultured ASM cells, TNFα, alone or in combination with other cytokines, such as IL-1β or IL-13, has been shown to stimulate the expression of various "pro-asthmatic" mediators, including cytokines (IL-6), chemokines (eotaxin, Rantes, IL-8) as well as adhesion molecules (ICAM-1, VCAM-1) (Halayko and Amrani, 2003). In addition, TNFα also cooperates with type II IFNγ to synergistically induce different pro-inflammatory proteins such as RANTES (John et al., 1997), fractalkine (Sukkar et al., 2004), or CD38 (Tliba et al., 2006), although the underlying mechanisms of such cooperation have not been elucidated. In that regard, we made the interesting observation that induction of defined genes including RANTES as well as CD38 by TNFα occurred, at least in part, via the secretion and autocrine action of endogenous IFNβ (Tliba et al., 2003, 2004). We were surprised to find that endogenous IFNβ acted as a negative regulator of IL-6 production induced by TNFα (Tliba et al., 2003). This suggests that, in addition to exerting cooperative effects, IFNs also induce antagonistic effects on TNFα-inducible genes. This hypothesis is supported by other reports in ASM showing that exogenous IFNγ can suppress TNFα-inducible inflammatory genes including vascular endothelial growth factor (Wen et al., 2003), IL-17 receptor expression (Lajoie-Kadoch et al., 2006), and CD38 (Tliba et al., 2006). This is an important finding because a better understanding of the inhibitory mechanisms exerted by IFNγ on TNFα-inducible inflammatory genes may offer new insight into the design of alternative approaches for the treatment of airway inflammation in asthma.

The purpose of the present study was to assess the suppressive mechanisms of IFNγ on TNFα-inducible pro-asthmatic genes in human ASM cells. We found that IFNγ acts as a potent inhibitor of TNFα-inducible, NF-κB-sensitive genes, including IL-6, IL-8, and eotaxin. TNFα-induced NF-κB acetylation, transactivation, and HAT function were impaired by IFNγ, and HDAC activity was significantly enhanced. TSA, a specific HDAC inhibitor, was able to reverse IFNγ inhibitory effects on TNFα-inducible genes. We therefore propose that IFNγ could negatively regulate expression of pro-inflammatory genes induced by TNFα by impairing NF-κB function through transcriptional repression exerted by an increased HDAC activity.

Materials and Methods

Smooth Muscle Cell Culture and Characterization. Human ASM cell culture was performed as described previously (Tliba et al., 2003). Human trachea was obtained from lung transplant donors in accordance with the procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings.

Western Blotting. Immunoblot analysis for total NF-κB p65, total IKKβ, phosphorylated IKKβ and IKBα, and anti-acetyl-lysine was performed as described previously (Amrani et al., 1999). In brief, cells were lysed in buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.1% deoxycholate, 10 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 mM EDTA, 10 mM NaF, and 2 mM Na_3VO_4 for 20 min at 4°C. Postnuclear extracts were obtained by centrifugation of lysates at 14,000*g* for 10 min. Antibodies against total p65, total IKKβ, IκB (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated IKKβ (Cell Signaling Technology, Danvers, MA) or anti-acetyl lysine antibody (2 μg/ml, clone 4G12; Upstate Biotechnology, Chicago, IL) were used as indicated by the manufacturer's instructions. Immunoprecipitation using NF-κB p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as indicated by the manufacturer's instructions. Equal amounts of protein were analyzed by 4 to 12% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The membranes were blocked in 5% milk or 5% bovine serum albumin (anti-phospho-protein antibodies) in Tris-buffered saline for 1 h and then incubated overnight with the primary antibody of interest at 4°C. After incubation with the appropriate peroxidase-conjugated secondary antibody (Roche Applied Science, Indianapolis, IN), the bands were visualized by the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and autoradiographed.

Transfection of ASM Cells. Because most standard transfection methods yield poor transfection efficiencies for ASM cells (10%), here we have optimized a high-efficiency transfection technique (Tliba et al., 2006). This technique is an extension of electroporation, using the Nucleofector kit for primary smooth muscle (Amaxa Biosystems, Cologne, Germany), in which plasmid DNA is transfected directly into the cell nucleus. Transfection was performed according to the manufacturer's instructions, and the program used was U-25. Sixteen to eighteen hours after transfection, the media were changed with serum-free media for the next 24 h. This method, using green fluorescence protein-pmax control vector (Amaxa Biosystems), enabled us to reach a transfection efficiency of 70%.

SEAP and β-Galactosidase Assays. To monitor NF-κB-dependent gene expression, ASM cells were cotransfected with κB-secreted alkaline phosphatase (SEAP) reporter vector and with pSV-$^{\beta}$-galactosidase vector used to normalize transfection efficiencies (Promega, Madison, WI). The activities of SEAP and β-galactosidase were evaluated using Great Escape SEAP detection kit (Clontech, Mountain View, CA) and β-galactosidase detection kit (Promega), respectively, according to the manufacturer's instructions.

RNA Isolation and Reverse Transcriptase PCR Analysis. Human ASM cells were serum-deprived in medium containing 0.1% fetal bovine serum for 24 h and exposed to 10 ng/ml TNFα to 1000 U/ml IFNγ and TNFα/IFNγ in combination for 24h. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Reverse transcriptase PCR analysis of IL-6, IL-8, and eotaxin was then performed as reported previously (Amrani et al., 2000). Primers used for IL-6: forward, 5′-CCAGCTATGAACTCCTTCTCCACAAGC-3′; reverse, 5′-GCTGGACTGCAGGAACTCCTTAAAGC-3′; IL-8: forward, 5′-ATGACT TCCAAGCTGGCCGTGGCT-3′; reverse, 5′-TCTCAGCCCTCTTCAAAAACT TCTC-3′ (Zhu et al., 2003); eotaxin: forward, 5′-GGATCCAACATGAAGGTCTCCG-3′, and reverse, 5′-GAATTCTTATGGCTTTGGAGTTGGAG-3′(Nie et al., 2005). The semiquantitative PCR approach was performed in parallel by investigating human GAPDH mRNA levels with the following primers: forward, 5′-ATGGATGATGATATCGCCGC-3′; reverse, 5′-TTAATGTCACGCACGATTTC-3′.

PCR was performed for 30 cycles at 94°C denaturation, 60°C annealing, and 72°C extension using *Taq* DNA polymerase (Pro-

mega). Reaction products were confirmed on 1% agarose (Fisher Biotech, Fair Lawn, NJ) gels with size markers (New England Biolabs, Ipswich, MA) and stained with ethidium bromide. The intensity of density area was analyzed using a Gel-Pro Analyzer (Silver Spring, MD). The final PCR product was expressed as the ratio to Actin used for scanning analysis.

NF-κB p65/DNA Interaction. Nuclear extraction was performed as described previously (Tliba et al., 2003, 2006). Five micrograms of nuclear extract wastested for p65-DNA binding activity by using TransAM NF-κB p65 kit, using a wild type sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and a specific NF-κB p65 antibody, according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The results (optical density measured at 450 nm) were expressed as percentage increase over basal (untreated cells).

Total Acetylation and Deacetylation Analyses. Nuclear extracts derived from cytokines treated or control cells were prepared as described previously (Tliba et al., 2003, 2006). Fifty micrograms of nuclear extracts were used for assessing HAT and HDAC activities according to the manufacturer's instructions [Oxford Biomedical (Oxford, MI) and Upstate Biotechnology, respectively].

Materials and Reagents. Tissue culture reagents and primers used for PCR were obtained from Invitrogen (Carlsbad, CA). Human rTNFα was provided by Roche Applied Science (Indianapolis, IN). rIFNγ, was purchased from R&D Systems. IKKβ inhibitor (BMS-345541) and HDAC inhibitor trichostatin A (TSA) were purchased from Calbiochem (San Diego, CA). Cytokine concentrations in the culture media were determined by enzyme-linked immunosorbent assay (Tliba et al., 2003).

Statistical Analysis. To compare differences between treatment means (expressed as mean ± S.E.), all data were subjected to one- or two-way analysis of variance when experiments were of a factorial design. After analysis of variance, Fisher's method of protected least significant differences was used as a multiple comparison test. Comparison of two populations was made with Student's t test. Values of $P < 0.05$ were sufficient to reject the null hypothesis for all analyses.

Results

IFNγ Suppressed TNFα-Induced Expression of Proinflammatory Genes. In previous reports, we made the surprising finding that TNFα, via the autocrine action of secreted IFNs, differentially modulates the expression of a number of pro-inflammatory genes, resulting in either the suppression of IL-6 or the enhancement of RANTES (Tliba et al., 2003). Here, we investigated the underlying mechanisms involved in the suppressive effect of IFNγ on TNFα-inducible inflammatory genes. Cells stimulated with TNFα, IL-6, IL-8, and eotaxin expression were significantly increased at 24 h ($P < 0.001$) (Fig. 1). In the presence of increasing concentrations of exogenous IFNγ (1–1000 U/ml), TNFα-induced ex-

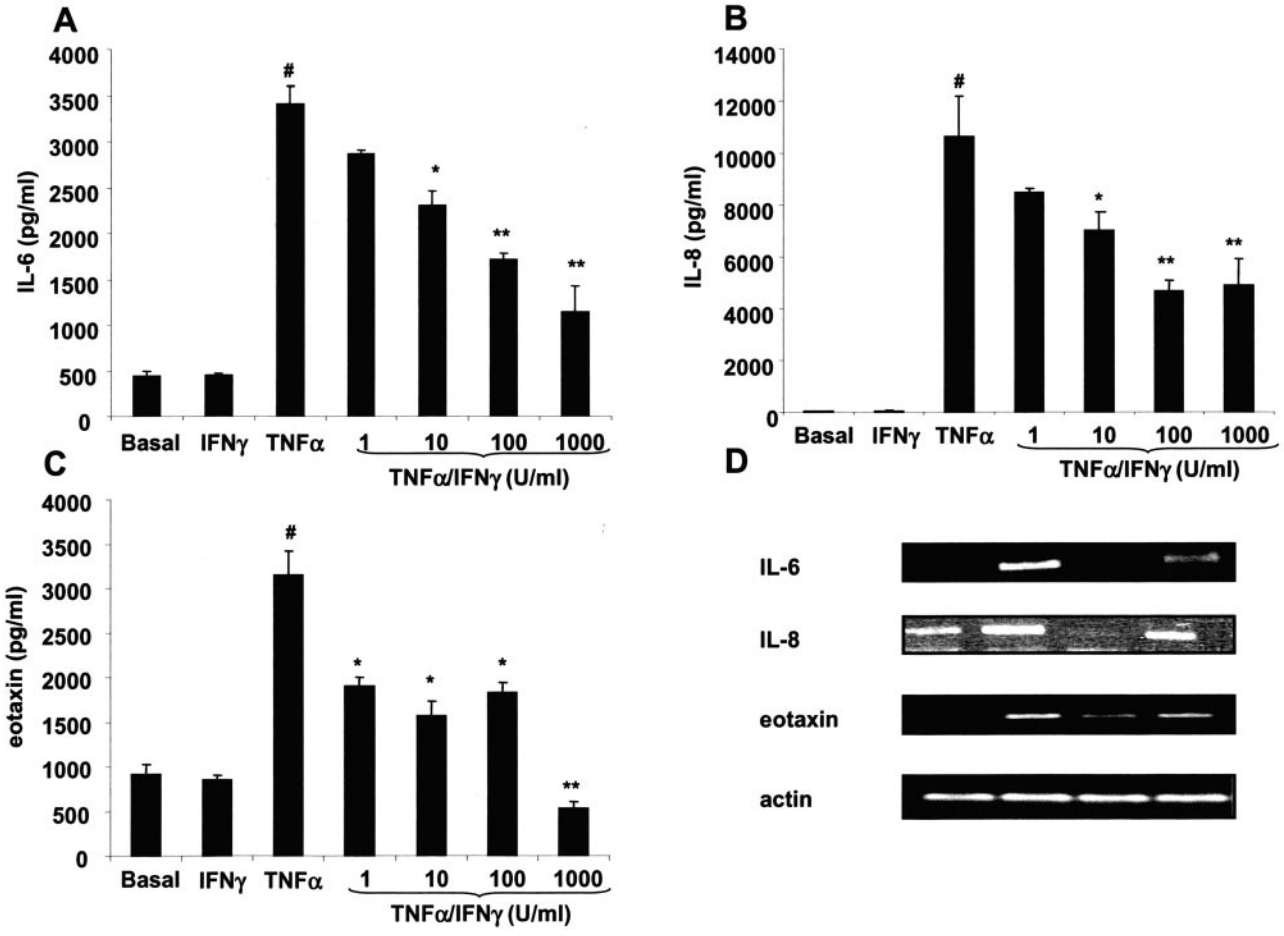

Fig. 1. IFNγ inhibited TNFα-induced IL-6, IL-8, and eotaxin expression. ASM cells were incubated with 10 ng/ml TNFα, with 1000 U/ml IFNγ and with both cytokines at indicated concentrations of IFNγ for 24 h. IL-6 (A), IL-8 (B), and eotaxin (C) gene expression were measured. The results are expressed in picograms per milliliter ± S.E.M. of three separate experiments. #, $P < 0.001$ compared with basal condition; ∗, $P < 0.05$ compared with cells treated with TNFα alone; ∗∗, $P < 0.001$ compared with cells treated with TNFα alone. D, cells were lysed, total mRNA was extracted, and reverse transcription-PCR was performed using specific IL-6, IL-8, and eotaxin primers, as described under *Materials and Methods*, under the same conditions (basal, TNFα, IFNγ, TNFα/IFNγ). The results show representative gels of three experiments for each gene.

pression of IL-6 and IL-8 was partially inhibited in a dose-dependent manner ($P < 0.01$, Fig. 1, A and B), whereas induction of eotaxin by TNFα was completely abrogated ($P < 0.001$) (Fig. 1C). Increasing TNFα concentrations did not affect the efficacy of IFNγ to suppress TNFα-inducible genes (data not shown). Reverse transcription-PCR analyses revealed that IFNγ exerted similar inhibitory effects at the gene levels with induction of IL-6, IL-8, and eotaxin by TNFα reduced by 80% ($P < 0.001$; Fig. 1D), 70% ($P < 0.001$; Fig. 1D) and 50% ($P < 0.01$; Fig. 1D) by IFNγ, respectively. These data demonstrate that IFNγ significantly inhibited TNFα-induced IL-6, IL-8, and eotaxin at both the protein and the mRNA levels. TNFα/IFNγ combination did not alter cell viability as assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown).

IKKβ Inhibitor BMS-345541 Suppressed TNFα-Induced Expression of Inflammatory Genes. Although multiple NF-κB binding sites are present in the promoters of different genes (Baldwin, 1996), few reports have examined the putative role of NF-κB pathways in the regulation of inflammatory genes in human ASM cells. Using the selective inhibitor of IKKβ BMS-345541 ($IC_{50} = 0.3$ μM) (Burke et al., 2003), we found that TNFα-induced expression of IL-6, IL-8, and eotaxin was suppressed in a dose-dependent manner (1–30 μM) reaching the basal level (Figs. 2 A–C; $P < 0.001$). The specificity of BMS-345541 concentrations was confirmed using a NF-κB reporter plasmid. As shown in Fig. 5A, TNFα induced a time-dependent (6–24 h) increase in NF-κB reporter activity that was completely abrogated in cells pretreated with the IKKβ inhibitor BMS-345541. We were also interested to find that IFNγ inhibited TNFα-induced NF-κB reporter activity in a concentration-dependent manner (Fig. 3B). Together, these results show that IKKβ plays an important role in mediating TNFα-induced gene expression and raises the hypothesis that IFNγ interferes with TNFα-induced gene expression by suppressing the NF-κB pathway.

IFNγ Treatment Had No Effect on Either TNFα-Induced IKKβ Phosphorylation or IκB Degradation. To

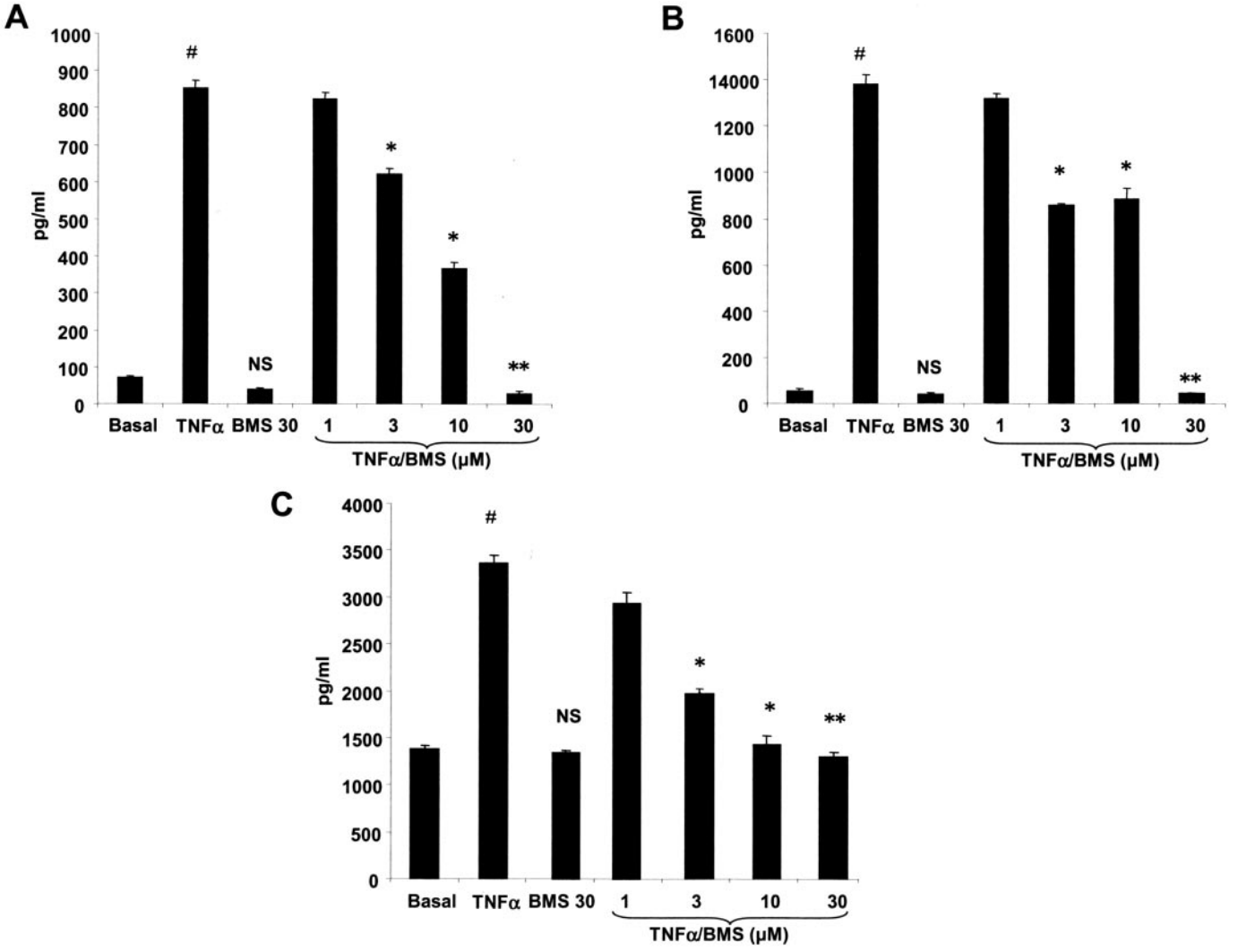

Fig. 2. The IKKβ inhibitor suppressed TNFα-induced IL-6, IL-8, and eotaxin expression. A, ASM cells were treated with TNFα (10 ng/ml) in the presence or absence of IKKβ inhibitor (BMS-345541) added alone at 30 μM or 1h before TNFα stimulation at the indicated concentrations (ranging from 1 to 30 μM). IL-6 (A), IL-8 (B), and eotaxin (C) expression was assessed by enzyme-linked immunosorbent assay as described under *Materials and Methods*. The results are expressed in picograms per milliliter of three separate experiments. #, $P < 0.001$ compared with basal condition; *, $P < 0.05$ compared with cells treated with TNFα alone; **, $P < 0.001$ compared with cells treated with TNFα alone; NS, nonsignificant compared with untreated cells.

further study the mechanisms by which IFNs suppressed TNFα-induced NF-κB-dependent gene expression, we examined the effect of IFNγ on NF-κB upstream activating cascades, including activation of IKKβ, degradation of IκBα, and NF-κB nuclear translocation and DNA binding capabilities using the TransAM technology (Active Motif, Carlsbad, CA). As shown in Fig. 4, IFNγ pretreatment failed to prevent TNFα-induced IKKβ phosphorylation (Fig. 4A), IκBα degradation (Fig. 4B), NF-κB DNA binding activity assessed at 2 and 24h (Fig. 4C), and TNFα-induced p65 accumulation in the nucleus (Fig. 4D). IFNγ alone failed to activate any of these signaling pathways. These results show that the mechanisms underlying the inhibitory effect of IFNγ on cytokine-induced NF-κB–dependent gene expression occurs at the nucleus, most likely on the transcriptional machinery.

IFNγ Regulated TNFα-Associated HAT Activity and NF-κB p65 Acetylation. Acetylation of proteins is an important step that regulates many important cellular events including transcriptional regulation of genes. Acetylation of histone, through the activity of HATs, allows gene transcription by opening the chromatin, whereas deacetylation of histone, which is dependent on deacetylases (HDAC), closes the chromatin and represses the transcription (Rahman et al., 2004; Barnes et al., 2005). We found that in cells treated with TNFα, HAT activity was increased by 98 and 159% at 2 and 24 h compared with basal. IFNγ alone modestly increased HAT activity and significantly suppressed TNFα-induced HAT activity (Fig. 5A). We also found that TNFα induced p65 acetylation, which is important for the NF-κB–dependent gene transcription (Hoberg et al., 2006). It is noteworthy that IFNγ inhibits TNFα-inducible NF-κB p65 acetylation (Fig. 5B).

HDAC Activity Was Synergistically Induced by TNFα/IFNγ Combination. Because IFNγ inhibits HAT activity induced by TNFα, we next examined whether IFNγ modulated HDAC activity. Our results demonstrated that, whereas TNFα or IFNγ separately modestly activated HDAC activity, the combination of both cytokines leads to a synergistic induction of HDAC activity (Fig. 6). It is noteworthy that the inhibitory effects exerted by IFNγ on TNFα-induced NF-κB-dependent gene expression (Fig. 7A), as well as IL-6 and IL-8 (Fig. 7, B and C), could be reversed by pretreating cells with TSA, a well characterized HDAC inhibitor (Barnes et al., 2005). These results suggest that modulation of HDAC function by both IFNγ/TNFα is playing a key role in the regulation of NF-κB-dependent gene expression.

Discussion

Growing evidence supports the notion that ASM could play a critical role in asthma by its ability to secrete a variety of pro-inflammatory mediators (reviewed in Halayko and Amrani, 2003). Therefore, identifying the factors and/or signaling pathways that regulate the secretion of inflammatory cytokines could provide new therapeutic options for the treatment of airway inflammation. Here, we found that IFNγ dose-dependently suppressed the expression of various "proasthmatic" genes, IL-6, IL-8, and eotaxin induced by TNFα by acting at the transcriptional level. More specifically, IFNγ was shown to abrogate TNFα-induced NF-κB–dependent gene transcription by modulating HDAC function. Our data demonstrate that targeting HDAC in ASM could represent a novel therapeutic approach to suppress expression of inflammatory genes.

In ASM cells, several reports showed that IFNγ/TNFα was an effective combination to synergistically induce different inflammatory genes including RANTES (John et al., 1997), COX-2 (Singer et al., 2003), CXCL10 (Hardaker et al., 2004), Fractalkine (Sukkar et al., 2004), or CD38 (Tliba et al., 2006). It is noteworthy that we found here that IFNγ can also act as a potent inhibitor of TNFα-induced expression of other genes

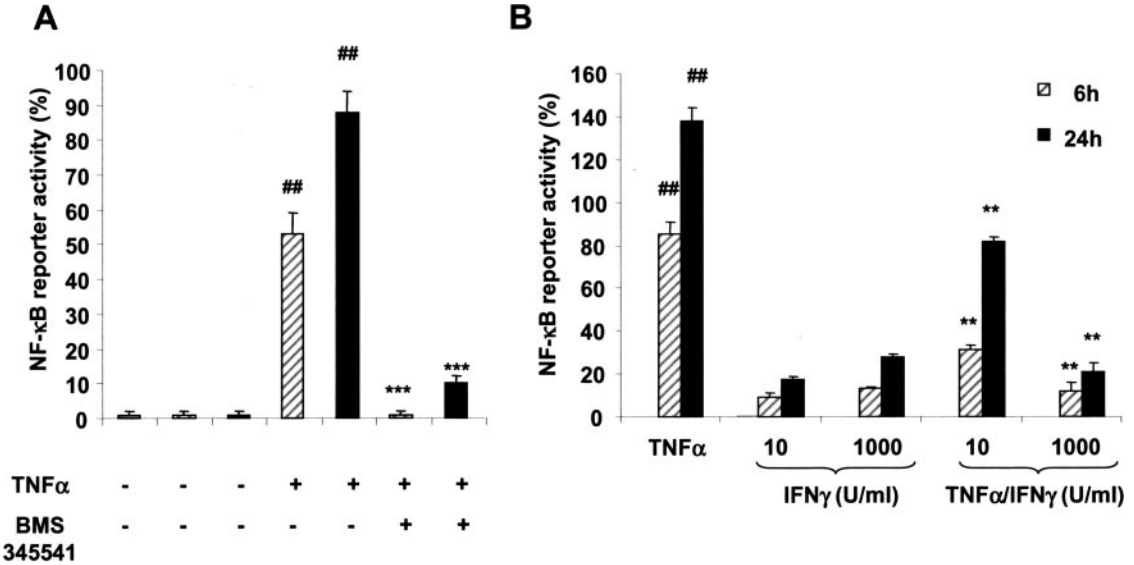

Fig. 3. Both IκKβ inhibitor and IFNγ suppressed TNFα-induced NF-κB-dependent gene expression. ASM cells were stimulated with TNFα (10 ng/ml), and the supernatant was taken for 6 and 24 h. Cells were transfected with SEAP reporter construct driven by a κB site as described under *Materials and Methods* to assess NF-κB gene expression. A, cells were treated with IKKβ inhibitor (BMS-345541, 30 μM) 1h before TNFα stimulation. B, cells were treated with 10 ng/ml TNFα alone or in combination with IFNγ (10 and 1000 U/ml). Data are representative of three separate experiments. #, $P < 0.01$ compared with basal condition; ##, $P < 0.001$ compared with basal condition; ∗, $P < 0.05$ compared with cells treated with TNFα alone; ∗∗, $P < 0.01$ compared with cells treated with TNFα alone; ∗∗∗, $P < 0.001$ compared with cells treated with TNFα alone.

including IL-6, IL-8, and eotaxin (Fig. 1). Thus, our study demonstrates that IFNγ/TNFα combination could result in either a cooperative or antagonistic expression of inflammatory genes in ASM cells. This IFNγ/TNFα antagonism in ASM cells has been described previously by two reports showing that IFNγ can also suppress other TNFα-inducible inflammatory genes, including vascular endothelial growth factor (Wen et al., 2003) and IL-17 receptor expression (Lajoie-Kadoch et al., 2006). Although studies in other cell types, including airway epithelial cells (Matsukura et al., 2003), dermal (Miyamasu et al., 1999) and corneal fibroblasts (Fukuda et al., 2002) demonstrated that IFNγ/TNFα combination leads to similar effects (i.e., reduced cytokine production), others found opposite results in which IFNγ treatment enhanced IL-6 and IL-8 expression in response to TNFα (van Wissen et al., 2002). Surprisingly little is known about the molecular mechanisms mediating IFNγ differential effects on gene expression in ASM as well as in other cell types, but our study demonstrates the IFNγ/TNFα antagonism probably occurs at the transcriptional level via the modulation of NF-κB pathways.

The NF-κB transcription factor is a vital regulator of cel-

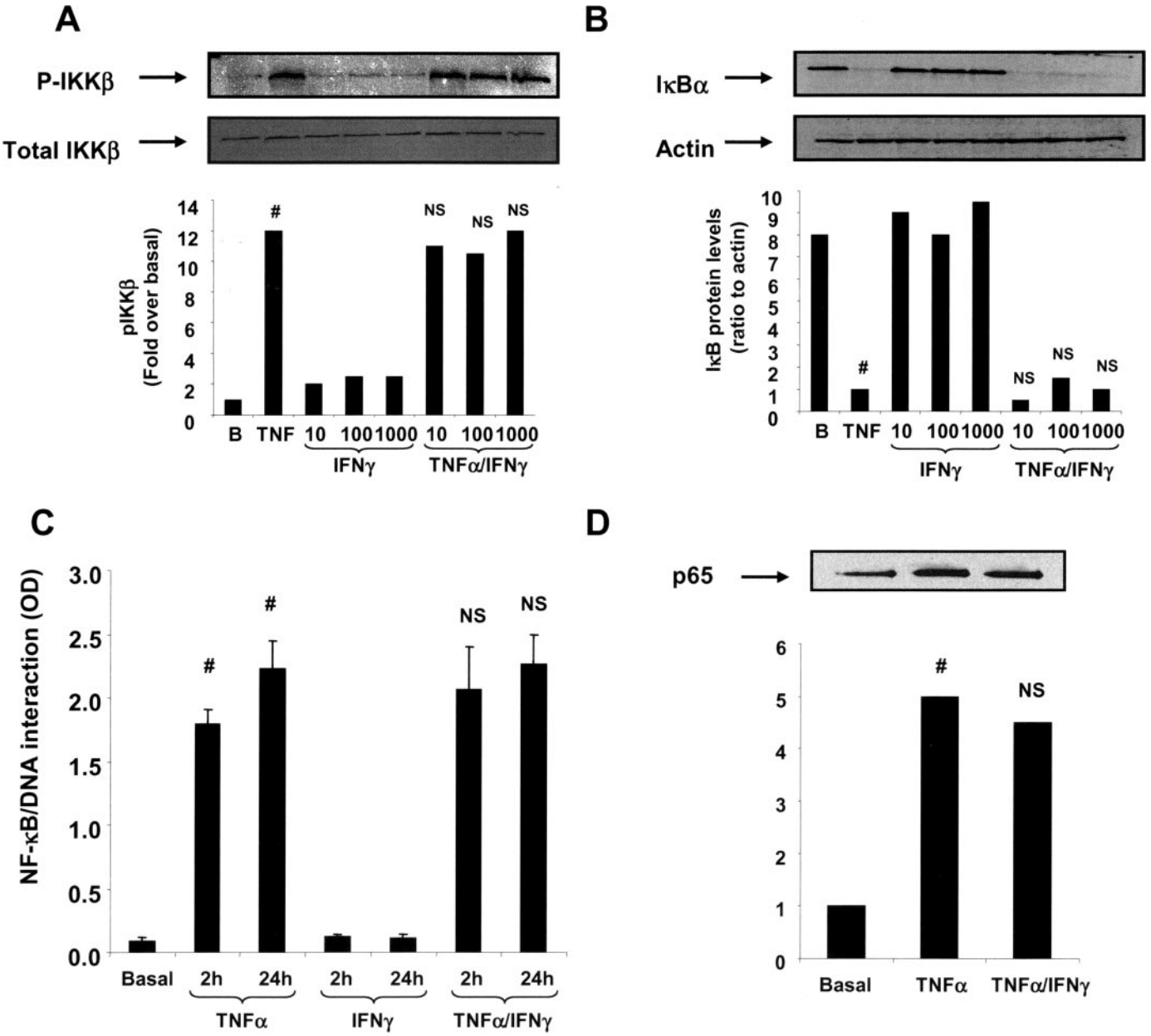

Fig. 4. IFNγ failed to prevent TNFα-induced IKKβ phosphorylation (A), IκBα degradation (B), NF-κB p65/DNA interaction (C), or NF-κB p65 nuclear translocation (D). Cells were stimulated for 24 h with TNFα (10 ng/ml), IFNγ, or the combination at the indicated concentrations of IFNγ and lysed, and total cell lysates were prepared for either IKKβ phosphorylation or IκB degradation by immunoblot analysis as described under *Materials and Methods*. Top, scanning of a representative gel; bottom, densitometry analysis with each value normalized over the mean density of the corresponding control (either total IKKβ or actin). C, cells were stimulated with TNFα (10 ng/ml), IFNγ, or the combination at the indicated time (2 or 24 h) and lysed, and nuclear extracts were prepared for NF-κB p65/DNA interaction using TransAM kit as described under *Materials and Methods*. The results are expressed as -fold increase over basal. D, immunoblot analysis was performed using p65 antibody on nuclear extracts of cells stimulated with TNFα or TNFα/IFNγ for 2 h as described under *Materials and Methods.* #, $P < 0.001$, compared with untreated cells (basal); NS, nonsignificant compared with cells treated with TNFα alone.

lular processes involved in immune response, cellular proliferation, differentiation, and apoptosis (Baldwin, 1996). Recent evidence using newly developed pharmacological inhibitors identified the necessary NF-κB-activating kinase, IKKβ, as a potential new target for treatment of inflammatory responses in asthma (Birrell et al., 2005). We and others showed that NF-κB is activated by TNFα or IL-1β in ASM cells and represents an essential transcription factor involved in the regulation of VCAM-1 (Issa et al., 2006), ICAM-1 (Amrani et al., 1999), CXC chemokine growth-related oncogene protein-α (Issa et al., 2006), and granulocyte macrophage–colony-stimulating factor (Lalor et al., 2004). Through the use of BMS-345541, a potent IKKβ inhibitor (Burke et al., 2003), we demonstrated here that NF-κB also played a central role in IFNγ-sensitive, TNFα-inducible genes IL-6, IL-8, and eotaxin. Using a reporter plasmid containing multiple κB enhancer elements (Fig. 3), we found that IFNγ almost completely abrogated TNFα-induced NF-κB-dependent gene expression. Combined together, these observations strongly suggest that IFNγ suppressed TNFα-associated genes by impairing NF-κB transactivation. It is noteworthy that some reports, but not all, showed the ability of type I or II IFNs (IFNα or IFNγ) to inhibit TNFα-induced NF-κB pathways in different cell types, including 2fTGH fibroblasts (Ganster et al., 2005), Ewin's sarcoma EW-7 cells (Sanceau et al., 2002), and Jurkat T cells (Manna et al., 2000). We found that the inhibition of cytokine-induced NF-κB transactivation by IFNγ most likely occurs at the transcriptional level because IFNγ failed to block upstream signaling events such as phosphorylation of IKKβ, degradation of the cytosolic inhibitor IκBα, as well as NF-κB nuclear translocation and NF-κB-DNA binding interaction (Fig. 4). Only one study performed in ME-180 cervical cancer cells demonstrated that IFNγ sensitized cells to apoptosis induced by TNFα. This occurs via the suppression of NF-κB-dependent gene transcription, an effect that was mimicked by overexpressing IRF-1, another member of IFN signaling (Suk et al., 2001). Our findings differ from previous observations made in other cell types that IFN blocks of NF-κB pathways induced by TNFα through multiple mechanisms, including 1) inhibition of NF-κB-DNA interaction (Sanceau et al., 2002), 2) prevention of IκBα degradation (Manna et

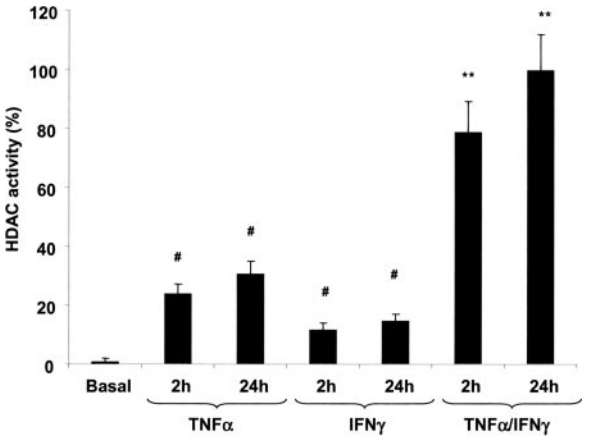

Fig. 6. IFNγ increased TNFα-induced HDAC activity. Cells were stimulated with TNFα (10 ng/ml), IFNγ, or the combination at the indicated time (2 or 24 h) and lysed; nuclear extracts were prepared for HDAC activity using a kit as described under *Materials and Methods*. The results are expressed as NF-κB reporter activity increase over values for untreated cells. #, $P < 0.005$ compared with untreated cells (basal); **, $P < 0.005$ compared with cells treated with TNFα alone.

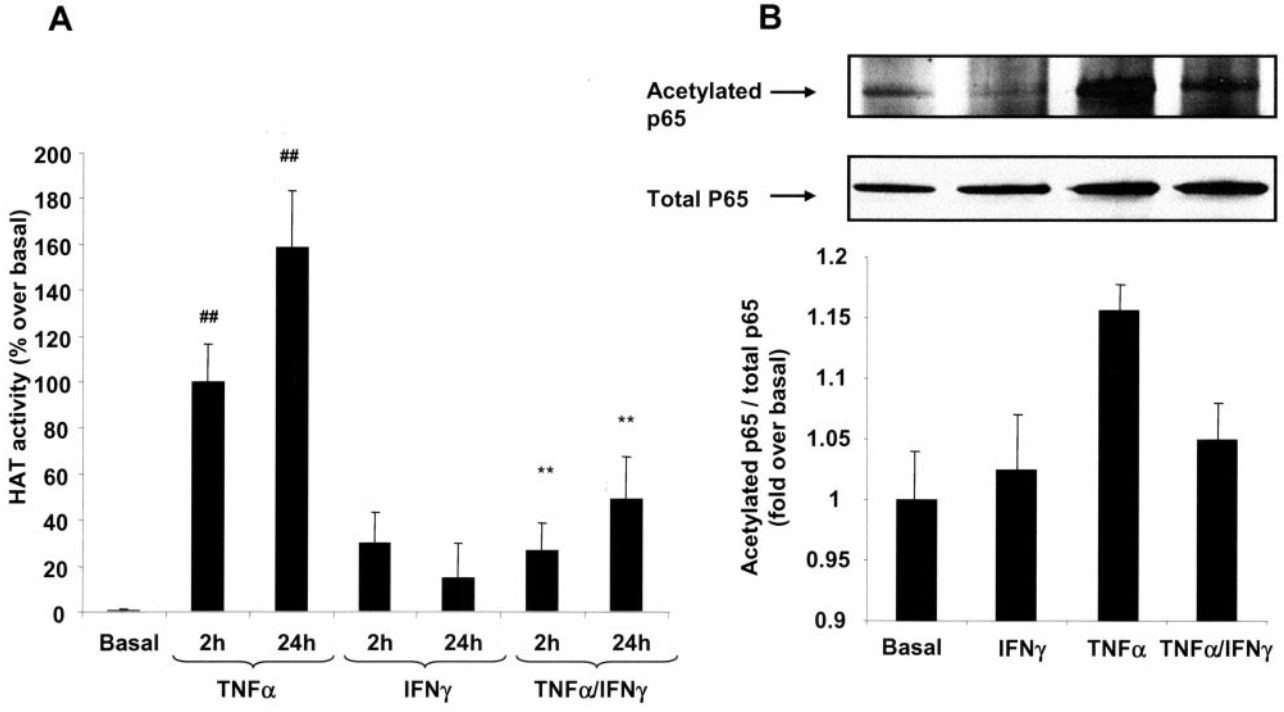

Fig. 5. IFNγ inhibited TNFα-induced histone acetyltransferase activity (A) and NF-κB p65 acetylation (B). A, cells were stimulated with TNFα (10 ng/ml), IFNγ, or the combination at the indicated time and lysed; nuclear extracts were prepared for HAT activity as described in *Materials and Methods*. B, cells stimulated with TNFα, IFNγ, or the combination for 2 h were lysed, immunoprecipitated with anti-p65 antibody, and assayed for anti-acetyl lysine by immunoblot analysis (gradient 4–12% SDS-PAGE). The results are expressed as increase over basal. #, $P < 0.005$ compared with untreated cells (basal); ##, $P < 0.001$ compared with untreated cells (basal); **, $P < 0.005$ compared with cells treated with TNFα alone.

al., 2000), or 3) tight regulation of TNFα receptor 1 activity induced by direct interaction with STAT1 (Wang et al., 2000; Wesemann and Benveniste, 2003). The molecular mechanisms by which IFNγ/TNFα combination leads to the suppression of NF-κB-inducible genes in ASM cells seem to be highly complex and distinct from other cell types. The present report presents the novel hypothesis that IFNγ/TNFα-induced change in protein acetylation plays an important role in the regulation of inflammatory genes in ASM cells.

Transcription of eukaryotic genes is complex and depends on different coactivators, such as p300, pCAF, and cAMP response element-binding protein-binding protein, as well as HATs and HDACs. HATs are responsible mainly for destabilizing the chromatin structure to allow accessibility of different transcription factors, including NF-κB, to the transcriptional site in the DNA. In contrast, HDACs serve as corepressors of gene transcription by restoring the condensation of DNA in the chromatin (Barnes and Karin, 1997). As reported in A549 cells (Rahman et al., 2004), we found that HAT activity is increased in ASM cells after TNFα treatment, an effect that was sustained up to 24 h (Fig. 5A). It is logical to assume that this prolonged HAT activity would be required to ensure the time-dependent induction of the inflammatory genes IL-6, IL-8, and eotaxin previously reported in ASM cells (Pang and Knox, 2001; Ammit et al., 2002). In agreement with our observation, Nie et al. (2005) reported that TNFα did induce histone H4 acetylation and the recruitment of NF-κB at the eotaxin promoter in human ASM cells. Studies using trichostatin A, a specific inhibitor of HDACs (Barnes et al., 2005), confirmed the current hypothesis that histone acetylation is an essential event for mediating TNFα-associated NF-κB-dependent genes (Ashburner et al., 2001; Adam et al., 2003). This assumption is further supported in our study by two unexpected observations: 1) HDAC activity was significantly increased in cells treated with IFNγ/TNFα combination and 2) TSA prevented the inhibitory actions of IFNγ on TNFα responses (NF-κB activation and IL-6 and IL-8 expression). Our results showed that p65 acetylation, which is important for the NF-κB-dependent gene transcription (Hoberg et al., 2006), was also enhanced by TNFα. We found that IFNγ decreases p65 acetylation induced by TNFα (Fig. 5B). Thus, the p65 acetylation level, because of the HAT/HDAC activities, is probably a part of the mechanisms by which IFNγ inhibits TNFα-inducible genes. Together, these data support the novel concept that changes in histone acetylation through the modulation of HDAC function may play an important role in the anti-inflammatory actions of

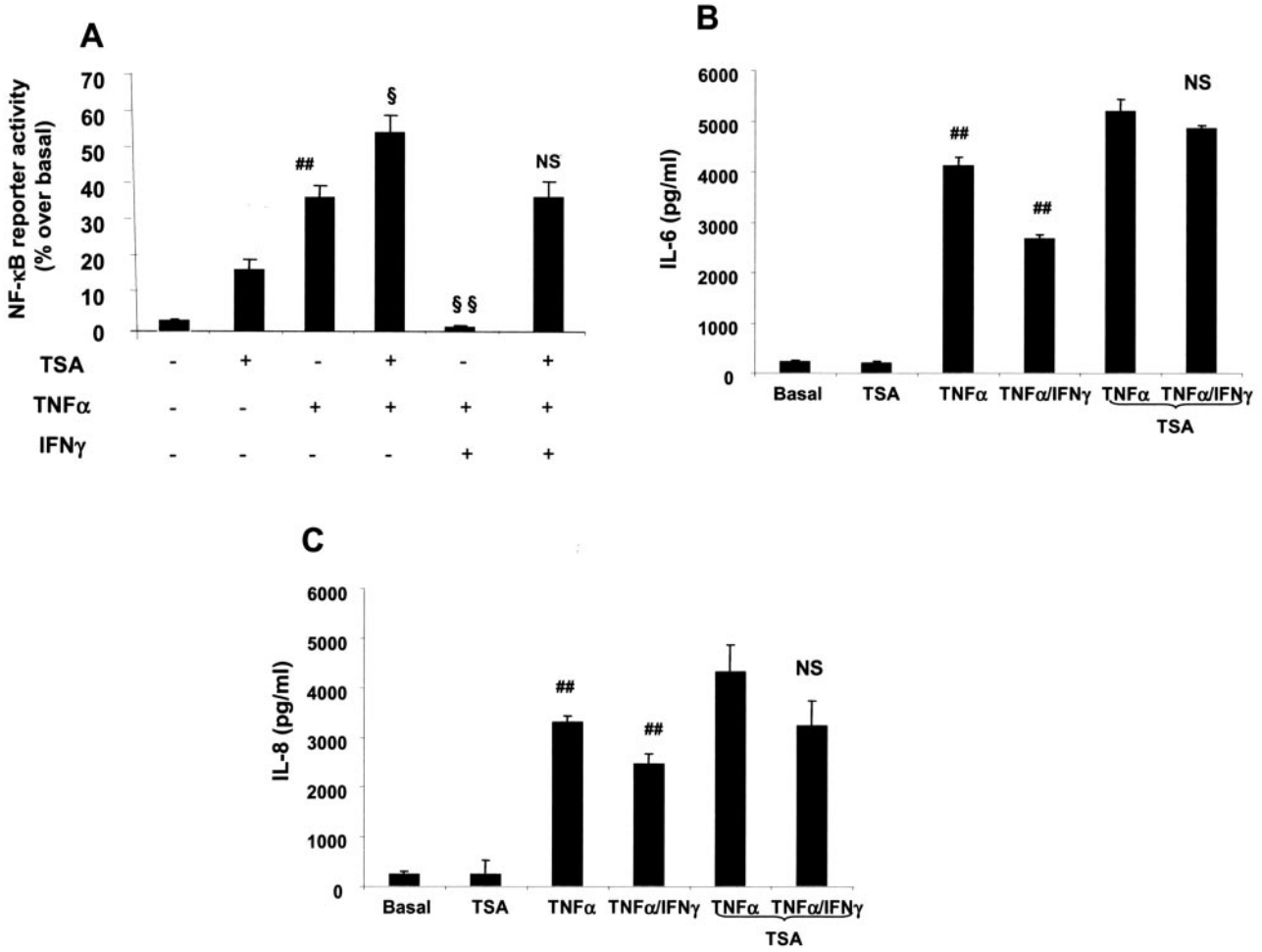

Fig. 7. HDAC inhibitor (TSA) restored the inhibitory effect of IFNγ on TNFα-induced NF-κB (A), IL-6 (B), and IL-8 (C) expression. Cells were stimulated with 400 nM TSA 1 h before TNFα (10 ng/ml), IFNγ (1000 U/ml), or the combination for 6 h. TSA completely prevented the inhibitory effect of IFNγ on TNFα-induced NF-κB (A), IL-6 (B), and IL-8 (C). ##, $P < 0.001$ compared with untreated cells (basal); §, $P < 0.05$ compared with cells treated with TNFα alone; §§, $P < 0.01$ compared with cells treated with TNFα alone; NS, not significant.

IFNs. Our report is the first to show that HDAC function can be modulated by TNFα/IFNs combination, although the nature of HDAC involved as well as the deacetylated proteins involved are unknown. In contrast to the unexplored role of HDAC in TNFα signaling, HDAC1 activity has been shown to be required for the complete transcription of IFN-inducible genes (Nusinzon and Horvath, 2003). Whether HDAC1 plays any role in the "selective" anti-inflammatory effects of IFNs remains to be determined. The essential question that should be raised is the nature of the synergistic signals leading to aberrant HDAC activity and suppression of NF-κB function.

In conclusion, in such diseases as asthma, an unbalance between HAT and HDAC activities could play an important role in the regulation of pro-inflammatory genes in ASM cells. It is noteworthy that subjects with severe asthma have reduced HDAC activity and increased HAT activity (Ito et al., 2002). Thus, a better understanding of the IFN mechanisms leading to increased HDAC function in the airways could provide new insight into the treatment of airway inflammatory diseases.

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