TNFα and IFNγ Synergistically Enhance Transcriptional Activation of CXCL10 in Human Airway Smooth Muscle Cells via STAT-1, NF-κB, and the Transcriptional Coactivator CREB-binding Protein

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Abstract

Asthmatic airway smooth muscle (ASM) expresses interferon-γ-inducible protein-10 (CXCL10), a chemokine known to mediate mast cell migration into ASM bundles that has been reported in the airways of asthmatic patients. CXCL10 is elevated in patients suffering from viral exacerbations of asthma and in patients with chronic obstructive pulmonary disease (COPD), diseases in which corticosteroids are largely ineffective. IFNγ and TNFα synergistically induce CXCL10 release from human ASM cells in a steroid-insensitive manner, via an as yet undefined mechanism. We report that TNFα activates the classical NF-κB pathway, whereas IFNγ activates STAT-1 and that inhibition of the JAK/STAT pathway is more effective in abrogating CXCL10 release than the steroid fluticasone. The synergy observed with TNFα and IFNγ together, however, did not lie at the level of NF-κB activation, STAT-1 phosphorylation, or in vivo binding of these transcription factors to the CXCL10 promoter. Stimulation of human ASM cells with TNFα and IFNγ induced histone H4 but not histone H3 acetylation at the CXCL10 promoter, although no synergism was observed when both cytokines were combined. We show, however, that TNFα and IFNγ exert a synergistic effect on the recruitment of CREB-binding protein (CBP) to the CXCL10, which is accompanied by increased RNA polymerase II. Our results provide evidence that synergism between TNFα and IFNγ lies at the level of coactivator recruitment in human ASM and suggest that inhibition of JAK/STAT signaling may be of therapeutic benefit in steroid-resistant airway disease.

Lung diseases such as asthma and COPD2 cause significant morbidity and mortality in Western societies. 5–10% of the asthmatic population are unresponsive to corticosteroids, the mainstay for asthma therapy, and patients with COPD are treated ineffectively with steroids. Additionally, viral exacerbations of asthma are a major cause of morbidity and mortality associated with asthma and are relatively unresponsive to steroids (1, 2).

CXCL10, a member of the CXC chemokine subfamily, is a potent chemoattractant for mast cells and T lymphocytes, cells implicated in the pathophysiology of asthma and COPD. CXCL10 is elevated in the airways of asthmatic patients and has been implicated in mast cell migration to the ASM bundles (3). CXCL10 also is elevated in the bronchial mucosa and bronchoalveolar lavage (BAL) fluid of subjects with moderate/severe asthma, which is largely steroid-resistant (4). Enhanced CXCL10 secretion also has been demonstrated in COPD (5–7), and more recently, it has been reported that serum CXCL10 levels are increased to a greater extent in asthmatics with acute virus-induced asthma compared with nonvirus-induced acute asthma, and increased levels are predictive of virus-induced asthma exacerbations (8). CXCL10 is released in response to IFNγ or TNFα from a number of inflammatory cell types (6, 9–12), and several reports also have shown that TNFα and IFNγ synergistically enhance CXCL10 release (13–16). Given the heightened cytokine milieu observed in severe asthma and COPD, understanding the mechanism for this increase clearly is a central question to address and requires an understanding of the mechanisms involved in transcription and translation of inflammatory products by ASM cells.

Transcriptional regulation involves transcription factors binding to recognition sequences in gene promoters (17–19). In quiescent cells, DNA is packaged tightly in chromatin, which must unravel to allow access of basal transcription factors and RNA polymerase II. Chromatin consists of four core histones, an H3-H4 tetramer and two H2A-H2B dimers that undergo covalent modifications (acetylation, phosphorylation, methylation), which control the unraveling process and thereby gene transcription (20–23). Our previous studies of CCL11 and CXCL8 have identified important transcription factors for these genes and, moreover, identified histone H4 acetylation as a key event in inflammatory gene transcription (18–19, 24–25). Little information is present regarding CXCL10 regulation by transcription factors and histone modifications in ASM. One study by Hardaker and colleagues (6) using pharmacological

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2 The abbreviations used are: COPD, chronic obstructive pulmonary disease; HASM, human airway smooth muscle; CBP, CREB binding protein; CREB, cAMP-response element-binding protein; ISRE, IFN-stimulated responsive element; AP, activator protein.
inhibitors implicates NF-κB in TNFα-induced CXCL10 release but did not study which transcription factors are utilized by IFNγ because although IFNγ produced a transient activation of NF-κB, no inhibitory action was observed with the NF-κB inhibitor used in the study. This study also highlighted a synergistic interaction between TNFα and IFNγ on CXCL10 release but did not explore the mechanism involved. TNFα also has been reported to cooperate with IFNγ to synergistically induce CCL5 (26), CX3CL1 (fractalkine, 27), and CD38 (28), although here, too, the underlying mechanisms are unclear.

Other potentially important transcription factors involved in CXCL10 regulation by IFNγ are STATs (signal transducers and activators of transcription). STATs are a family of latent cytoplasmic proteins that are activated when cells encounter various extracellular polypeptides, such as interferons and interleukins (29), and appear to be essential for responsiveness to IFNα and IFNγ (30–31). Ohmori and colleagues (32) reported that the frequently observed synergy between TNFα and IFNγ depends in part upon cooperation between STAT-1α and NF-κB in NIH3T3 fibroblasts and is likely mediated by their independent interaction with one or more components of the basal transcription complex. However, the authors but did not explore this possibility experimentally. IFNγ alone, or in combination with TNFα, also has been reported to involve p48, a transcription factor that complexes with STAT-1α and binds to interferon stimulated response element (ISRE) sites on the promoter. Induction of CXCL10 by TNFα/IFNγ also required NF-κB binding sites that bound p65 homodimeric NF-κB (15–16).

The aim of the present study was to characterize the transcription factors, histone modifications, and RNA polymerase II recruitment to the CXCL10 promoter to determine at which point of regulation synergism can be explained between TNFα and IFNγ. We show that TNFα activates the classical NF-κB pathway, IFNγ signals via STAT-1, and their synergism on CXCL10 gene induction is due not to their effects on transcription factor activation or histone modification, but rather a direct effect on the recruitment of the transcriptional coactivator CBP and RNA polymerase II. We also demonstrate that inhibition of the janus kinase (JAK)/STAT signaling pathway may be a novel approach to targeting steroid-resistant inflammatory lung disease. This is the first observation in primary airway cells and could highlight a novel anti-inflammatory therapy for the treatment of airway inflammation.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**—Polyclonal anti-human pJAK1, pJAK2, pSTAT-1α, STAT-1α, and RNA polymerase II antibodies were purchased from Cell Signaling Technology; anti-human p65 was from Santa Cruz Biotechnology; acetylated histone H3 and H4 was from Upstate Biotech, Inc.; JAK inhibitor 1 was from Calbiochem; recombinant human IFNγ and TNFα were from R&D Systems (Abingdon, Oxon, UK); MB120L and fluticasone propionate were kind gifts from GlaxoSmithKline; FuGENE 6 was from Roche Applied Science; the Dual-Luciferase reporter assay system, Renilla luciferase pRL-SV40 was from Promega; SYBR Green and Excite Master Mix for real-time PCR was from Biogene; primers were from MWG Biotech; ChIP-IT kits were from Active Motif; human and murine CXCL10 ELISA duosets and human and murine recombinant IFNγ and TNFα were from R&D Systems. All other reagents were purchased from Sigma.

The NF-κB-dependent luciferase reporter, 6NF-κBtkluc (33) was obtained from Dr. Newton (University of Calgary). The CXCL10 promoter-driven luciferase, mutation and deletion constructs has been described previously (34). In all experiments, empty vector experiments were performed, which had little or no significant effect on luciferase activity (data not shown).

**Cell Culture**—Human tracheas were obtained from three to six post-mortem individuals. Primary cultures of human ASM cells were prepared from explants of ASM according to methods reported previously (25, 35–37). This protocol was approved by the Nottingham City Hospital Research Ethics Committee. Cells at passages 6–7 were used for all experiments. We have shown previously that cells grown in this manner have the immunohistochemical and light microscopic characteristics of typical ASM cells.

**Experimental Protocol**—HASM cells were cultured to confluence in either six-well plates for Western blotting experiments or 24-well culture plates for other all experiments in a humidified, 5% CO2, 37 °C incubator using Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum (Seralab, Crawly Down, Sussex, UK), 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine, and 2.5 µg/ml amphotericin B (Sigma). The cells were growth-arrested in serum-free medium for 24 h prior to experiments. Immediately before each experiment, fresh serum-free medium containing cytokine or relevant vehicle was added. In concentration response experiments, cells were incubated for 24 h with 0.001–100 ng/ml TNFα or IFNγ. When cytokines were used in combination cells were stimulated with submaximal concentrations of each cytokine (0.1 ng/ml TNFα and 10 ng/ml IFNγ). Chemical inhibitors and steroids were incubated for 30–60 min before cytokine exposure as indicated in the figure legend. At the indicated times, the culture media were harvested and stored at −20 °C for subsequent ELISAs.

**CXCL10 Assay**—The enzyme-linked immunosorbent assay (ELISA) was used to measure CXCL10 (R&D Systems) according to the manufacturer’s instructions.

**RNA Isolation and Reverse Transcription**—Cells in six-well plates were treated with cytokines and collected at times indicated in the text. Total RNA was isolated by using the RNeasy mini kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol with on-column DNase digestion. 1 µg of total RNA was reverse transcribed in a total volume of 20 µl including 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 25 units of RNase inhibitor (Promega), 0.5 µg of oligo(dT)15 primer, 0.5 mM each dNTPs, and 1× first-strand buffer provided by Promega. The reaction was incubated at 42 °C for 90 min.

**Quantitative Real-time RT-PCR—** CXCL10 expression was determined using the following primer sequences: sense, 5′-GAAAATTATTCCTGCAAGCCAATTT-3′ and antisense, 5′-TCACCCCTTTTTCATGTAGCA-3′ (34). β2-Microglobulin was used validated for use in this assay and used as the
housekeeping gene. 1 ng of reverse-transcribed cDNA was subjected to real-time PCR using Excite Real-time Mastermix with SYBR Green (Biogene, Cambridge, UK) and the ABI Prism 7700 detection system (Applied Biosystems, Warrington, Cheshire, UK) as described previously (25). CXCL10 expression was normalized to the housekeeping gene by dividing the mean of the CXCL10 triplicate value by the mean of the $\beta_2$-microglobulin triplicate value. This was then expressed as fold increase over nonstimulated cells at each time point.

**Western Blot Analysis**—HASM cells were treated for times indicated in the text, cells were washed, and proteins were extracted as described previously (25). Anti-human pSTAT-1, total STAT-1, or pJAK1 and pJAK2 polyclonal antibodies were used at 1:1,000 dilution.

**CXCL10 mRNA Stability**—Serum-starved HASM cells were treated with or without TNFα (1 ng/ml), IFNγ (100 ng/ml), or TNFα (0.1 ng/ml) and IFNγ (10 ng/ml) in combination for 3 h before the addition of actinomycin D (5 μg/ml) for the times indicated to block new transcript generation. RNA was extracted and quantitative real-time RT-PCR of CXCL10 expression was measured as detailed above.

**Transient Transfection of CXCL10 Promoter-driven Luciferase Constructs**—All transient transfections were conducted by using FuGENE 6 according to the recommended protocol of the manufacturer and as described previously (25). A 1:3 ratio was used; 0.4 μg/well plasmid per 1.2 μl FuGENE 6, together with 0.8 ng/well of Renilla luciferase reporter/well as an internal control. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control Renilla luciferase activity. The fold increase was obtained by comparing relative luciferase activity from experiment groups against that from their respective controls. Data are expressed as fold difference, as little and no significant difference was seen in baseline levels observed between each construct when nonstimulated (data not shown). The transfection rate was between 25 and 30% as measured by transfection with a green fluorescent protein expression vector.

**Chromatin Immunoprecipitation Assay (ChIP)**—HASM cells were cultured to confluence in T75 cm² flasks, growth arrested, and incubated with media or cytokines for 60 min. The ChIP assay was performed using the ChIP-IT kit (Active Motif, Rixensart, Belgium) following the manufacturer’s protocol and as described previously (25). 4 μg of antibody or IgG control was used for each immunoprecipitation and stimulation parameter. IgG binding control levels did not change significantly from nonstimulated levels for each antibody tested indicating specificity of antibodies used (data not shown). The CXCL10 primers used yielded a 134-bp product corresponding to -224 to -90 of the CXCL10 gene promoter and were as follows: forward, 5’-TTTGAAAAGTGAACCTAAATTC-3’ and reverse, 5’-CAGGAACGACAGCGGTTT-3’.

**Cell Viability**—Drug and vehicles toxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously (35). None of the drugs/vehicles altered cell viability (data not shown).

**Statistical Analysis**—To determine whether there was synergy between TNFα and IFNγ, the sum of TNFα (0.1 ng/ml) and IFNγ (10 ng/ml) alone was compared with TNFα and IFNγ, Wilcoxon signed rank test was used to determine differences, and a p value = 0.05 was assumed to be significant. For all other experiments, statistical analysis was performed on the absolute data using a t test with a Mann-Whitney posttest for single comparisons and a Kruskal-Wallis test with a Dunn’s post-test for multiple comparisons. A plus sign (+) represents a significant (p < 0.05) differences from the nonstimulated control group; and the number sign represents a significant (p < 0.05) difference from stimulated control group.

**RESULTS**

**TNFα and IFNγ Synergistically and Transcriptionally Induce CXCL10 Release and mRNA**—In the absence of stimulus, HASM cells released an undetectable amount of CXCL10. TNFα and IFNγ (0.01–100 ng/ml for 24 h) concentration-dependently increased CXCL10 release from HASM cells (from 0 to 6886.3 ± 2403.3 pg/ml for TNFα and 248.7 ± 65.2 pg/ml for IFNγ each at 100 ng/ml; n = 3) (Fig. 1A). In all further experiments, CXCL10 release was stimulated using 1 ng/ml TNFα and 100 ng/ml IFNγ.

To explore synergy between TNFα and IFNγ, submaximal concentrations of each cytokine were chosen (10 ng/ml IFNγ and 0.1 ng/ml TNFα) to ensure that cytokine release was not supramaximal and could be modulated by pharmacological inhibitors in future experiments. Using these concentrations, IFNγ induced 61.9 ± 29.2 pg/ml, and TNFα induced 567.3 ± 197.5 pg/ml CXCL10. However, addition of these cytokines together synergistically induced CXCL10, inducing 19,920.9 ± 3454.8 pg/ml (Fig. 1A). These experiments were mirrored at the level of mRNA. After a 6-h stimulation, TNFα (1 ng/ml) and IFNγ (100 ng/ml) increased CXCL10 mRNA from 0 to 0.42 ± 0.14 and 0.096 ± 0.02, respectively. Synergy also was observed at the mRNA level as the mRNA level increased to 1.44 ± 0.07 in the presence of both cytokines (10 ng/ml IFNγ and 0.1 ng/ml TNFα; Fig. 1B, n = 3). Actinomycin D (5 μg/ml) addition indicated that the increase in CXCL10 mRNA observed was mediated transcriptionally (Fig. 1B).

**TNFα and IFNγ Do Not Regulate CXCL10 via Post-transcriptional Mechanisms**—Regulation of CXCL10 has been reported previously to be mediated via both transcriptional and post-transcriptional mechanisms (38); therefore, we investigated whether costimulation with TNFα and IFNγ affect CXCL10 mRNA stability. As can be seen in Fig. 1C, CXCL10 decay over time was not affected by TNFα or IFNγ alone, or when cells were costimulated with these cytokines, indicating post-transcriptional regulation does not play a role in this pathway. These data support that shown in Fig. 1B suggesting CXCL10 is regulated transcriptionally under these experimental conditions.

**TNFα and IFNγ Synergistically Enhance CXCL10 Promoter Activity**—TNFα (1 ng/ml) and IFNγ (100 ng/ml) increased luciferase activity 2.5 ± 0.2-fold and 4.5 ± 0.8-fold, respectively (Fig. 1D). To determine whether synergism also exists at the promoter level, transfected cells were stimulated with TNFα and IFNγ together (0.1 ng/ml TNFα plus 10 ng/ml IFNγ). This resulted in a 13.4 ± 3.4-fold increase in promoter activity, confirming that synergy lies at the transcriptional level (Fig. 1D).
TNFα and IFNγ Recruit CBP to the CXCL10 Promoter

FIGURE 1. TNFα and IFNγ induce CXCL10 transcriptionally. A, HASM cells were incubated with increasing cytokine concentrations (0.01–100 ng/ml) or both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) for 24 h, and CXCL10 was released into the supernatant measured by ELISA (mean ± S.E., n = 7 from independent donors). An asterisk indicates statistically significant synergism observed when cytokines used in combination, p value ≤ 0.05. B, for mRNA, cells were pretreated (30 min) with actinomycin D (5 μg/ml) prior to cytokine stimulation for 6 h. CXCL10 and β2-microglobulin mRNA was measured by real-time PCR, and the relative expression was calculated by dividing CXCL10 values by β2-microglobulin at the same data point (mean ± S.E., n = 3 from independent donors). C, serum-starved HASM cells were treated with or without TNFα (1 ng/ml), IFNγ (100 ng/ml), or TNFα (0.1 ng/ml) and IFNγ (10 ng/ml) in combination for 3 h before the addition of actinomycin D (5 μg/ml) for the times indicated to block new transcript generation. RNA was extracted, and quantitative real-time RT-PCR of CXCL10 gene expression was measured by real-time PCR, D50 to 60% confluent human ASM cells in 24-well plates were cotransfected with Renilla luciferase internal control reporter (0.8 ng/well), and the full-length CXCL10 promoter firefly luciferase reporter construct (0.4 μg/well) using FuGENE 6 for 18 h and then stimulated with or without TNFα (1 ng/ml) or IFNγ (100 ng/ml) and both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) for 6 h. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control Renilla luciferase activity. The results are expressed as mean ± S.E. of three separate experiments performed in quadruplicate. Statistical analysis was performed on the absolute data using a t test with a Mann-Whitney posttest for single comparisons and a Kruskal-Wallis test with a Dunn’s post-test for multiple comparisons. +, significant (p < 0.05) differences from nonstimulated control group; *, significant (p < 0.05) difference from stimulated control group (mean ± S.E.).
increase; raw values from 0.58 ± 0.1 to 1.79 ± 0.14 for TNFα, 0.71 ± 0.1 for IFNγ, and 1.35 ± 0.09 together; Fig. 3D). These data suggest that IFNγ-induced CXCL10 release is independent of NF-κB activation and that synergy observed with TNFα and IFNγ on CXCL10 release from HASM cells does not lie at the level of NF-κB activation and p65 binding.

**Synergy Does Not Lie at the Level of STAT-1α Activation**—IFNγ is known to activate the JAK/STAT signaling kinases to regulate gene production (44–45), hence, we probed this pathway in HASM cells. We first used a nonselective JAK inhibitor, JAK inhibitor 1. JAK inhibitor 1 concentration-dependently inhibited IFNγ-induced CXCL10 release, inhibiting release by 100% (from 336.1 ± 60.9 pg/ml to 0 pg/ml; n = 3), indicating that IFNγ is signaling via this pathway (Fig. 4A).

We then went on to determine which JAK and STAT isoforms are activated by IFNγ in HASM cells. IFNγ-induced phosphorylation of JAK2 and STAT-1α but not JAK1 phosphorylation, (Fig. 4B). To determine whether TNFα induced synergy via this pathway, we investigated the recruitment of STAT-1α to the CXCL10 promoter by each cytokine separately and in combination. IFNγ induced an 8.75 ± 1.8-fold increase in STAT-1α recruitment, whereas TNFα had little effect (1.63 ± 0.49-fold increase). Synergy was not observed when TNFα and IFNγ were added together (4.99 ± 1.5-fold increase; raw values from 0.031 ± 0.01 to 0.25 ± 0.01 for IFNγ, 0.045 ± 0.01 TNFα, and 0.13 ± 0.02 together; Fig. 4C).

We also tested the effects of the JAK inhibitor on the release of CXCL10 induced by both cytokines. TNFα and IFNγ-induced (1 and 10 ng/ml, respectively) CXCL10 release was abrogated by 65.9 ± 6.1% (from 6948.4 ± 1234.4 pg/ml to 2232.9 ± 260.6 pg/ml using 10−5 m Jak 1 inhibitor). Fluticasone was ineffective at reducing CXCL10 release under these conditions (Fig. 4D). These data suggest that inhibitors of the JAK/STAT pathway may prove more effective than a steroid in steroid-resistant disease.

**Essential Elements in the Human CXCL10 Promoter Region in Response to TNFα, IFNγ, and Both Cytokines in Combination**—We wanted to confirm the transcription factors involved in cytokine-induced CXCL10 gene transcription at a more molecular level, hence, we transfected HASM cells with luciferase reporter plasmids containing mutations and deletions of the wild-type CXCL10 promoter sequence (Fig. 5A) (34). Data are expressed as fold difference, as little and no significant difference was observed in baseline levels observed between each construct when nonstimulated (data not shown). TNFα (1 ng/ml) and IFNγ (100 ng/ml) increased luciferase activity 2.5 ± 0.2-fold and 4.5 ± 0.8-fold, respectively (Fig. 5, B and C). To determine whether synergism also exists at the promoter level, transfected cells were stimulated with TNFα and IFNγ together (0.1 ng/ml TNFα plus 10 ng/ml IFNγ). This resulted in a 13.4 ± 3.4-fold increase in promoter activity, which was significantly higher than either cytokine alone (Fig. 5D).

Next, we compared the responses to the full-length CXCL10 promoter to constructs within which each of the two proximal NF-κB recognition sites and the proximal ISRE individually mutated for TNFα and IFNγ, respectively. Compared with the native promoter, mutation of either of the NF-κB sites led to a significant reduction in promoter activity after stimulation with TNFα (Fig. 5B). A reduction also was observed when mutating the proximal ISRE binding site in the full promoter after stimulation with IFNγ (Fig. 5C) but not when neither NF-κB binding sites or the AP-1 binding site was mutated (data not shown). In keeping with either cytokine alone, mutation of the NF-κB and ISRE binding sites also abrogated promoter activity after stimulation with TNFα and IFNγ combined (Fig. 5D). These data confirm the pharmacological data suggesting TNFα-induced CXCL10 released is controlled by NF-κB, IFNγ-induced release by STAT transcription factor and that both play a role when both cytokines are added together. These data support that published by Ohmori and colleagues (32), whereby TNFα and IFNγ independently activated nuclear factors capable of specific interaction with the ISRE and NF-κB sites, respectively.

**TNFα and IFNγ Do Not Acetylate Histone H3 or Synergistically Induce Histone H4 Acetylation at the CXCL10 Promoter**—Given the lack of synergy observed at the cellular transcription factor activation level, we then postulated that both cytokines in combination could be having an indirect effect by modifying the chromatin environment. Histone H4 acetylation at the promoter was low basally, yet increased by both TNFα and IFNγ (13.6 ± 3.0-fold and 7.4 ± 1.4-fold, respectively; Fig. 6A). How-

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**FIGURE 2.** Effect of the steroid fluticasone on cytokine-induced CXCL10 release HASM cells grown on 96-well plates were growth arrested for 24 h and pretreated for 60 min with fluticasone (10−11 to 10−6 m) before stimulation with TNFα (1 ng/ml) (A), or IFNγ (100 ng/ml) (B), or both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) (C) for 24 h. CXCL10 released into the supernatant was measured by ELISA. Statistical analysis was performed on the absolute data using a t test with a Mann-Whitney post-test for single comparisons and a Kruskal-Wallis test with a Dunn’s post-test for multiple comparisons. +, significant (p < 0.05) differences from nonstimulated control group; *, significant (p < 0.05) difference from stimulated control group (mean ± S.E., n = 4 from independent donors). DMSO, dimethyl sulfoxide.
FIGURE 3. Effect of cytokines on NF-κB activation and p65 DNA binding

The data in would suggest that the level of synergy is not due
cytokines in combination (0.1 ng/ml TNF
0.05; IFN
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FIGURE 3. Effect of cytokines on NF-
pretreated for 30 min with ML120B (10
Relative luciferase activity was obtained by normalizing the firefly lucifer-
plates were cotransfected with
transient donors).

The
promoter when TNF
75-cm² dishes were incubated with TNF
both cytokines were added in combination, a 5.85 ± 0.29-fold increase in RNA polymerase II at the CXCL10 promoter was achieved (raw values of immunoprecipitated RNA polymerase II/input were as follows: control, 0.014 ± 0.003; TNFα, 0.029 ± 0.005; IFNγ, 0.29 ± 0.004; TNFα and IFNγ, 0.072 ± 0.013; Fig. 6D).

These data suggest that TNFα and IFNγ induce CXCL10 release synergistically via RNA polymerase II activation, which results in a heightening in gene transcription. This is the first report in primary airway cells.

CytoKine-induced CXCL10 Release Is Abolished by α-
Amanitin—To further investigate the regulation of CXCL10 by RNA polymerase II, we used the RNA polymerase II inhibitor α-amanitin (46). α-Amanitin (0.1 μg/ml) inhibited TNFα, IFNγ, and TNFα and IFNγ-induced CXCL10 release from HASM cells by 93.3 ± 2.8%, 96.6 ± 4.8%, and 81.8 ± 4.1%, respectively (Fig. 6E), confirming a role for RNA polymerase II in CXCL10 gene transcription.

DISCUSSION

The first aim of the current study was to find the mecha-
ism of the synergistic enhancement of CXCL10 release in
HASM cells after TNFα and IFNγ treatment. We found that
the synergy between TNFα and IFNγ on CXCL10 release
was not due to the combination of these agents causing
increased activity of the two transcription factors NF-κB and
STAT-1α that these two cytokines act through, respectively.

We also report that it is not due to increased binding of these transcription factors to the CXCL10 promoter or enhanced acetylation of histone H4, an epigenetic change that we have shown is responsible for the activation of other inflammatory gene promoters in these cells, such as CCL11 and CXCL8 (24–25). We found that TNFα and IFNγ synergisti-
cally recruited the coactivator CBP to the CXCL10 promoter
along with heightened recruitment of RNA polymerase II to
the promoter, suggesting that this was the main cause of
there synergistic effect on CXCL10 release.

Second, we wanted to determine the effect of transcription factor inhibitors in this steroid resistant model of airway inflammation, given the pathogenic role CXCL10 exerts in steroid-resistant airway disease, e.g. viral-induced asthma exacerbations and COPD (3, 8). We found that TNFα-induced
CXCL10 release was inhibited by both the steroid fliclasone

ever, TNFα and IFNγ only induced a 5.5 ± 1.1-fold increase in
binding (raw values of immunoprecipitated acetylated histone
H4/input were as follows; control, 0.03 ± 0.007; TNFα, 0.3 ±
0.05; IFNγ, 0.19 ± 0.02; and TNFα + IFNγ, 0.12 ± 0.01; Fig.
6A). We also report a lack of synergy on H4 acetylation at the
CXCL10 promoter when TNFα and IFNγ were used at 0.1
ng/ml and 10 ng/ml, respectively, confirming these data. No
acetylation of histone H3 at the CXCL10 promoter was
observed with either cytokine alone or in combination (Fig. 6B).

These data in would suggest that the level of synergy is not due
to modification of the histone H3 or H4 acetylation status at the
CXCL10 promoter.

TNFα and IFNγ Synergistically Recruit CBP and RNA Poly-
merase II to the CXCL10 Promoter—We have reported previ-
ously that TNFα recruits p300/CBP-associated factor to the
CCL11 promoter in a PKCβ-dependent manner in HASM cells;
therefore, we investigated the effects of TNFα and IFNγ on
p300, CBP, and p/CAF recruitment to the CXCL10 promoter
(25). Both cytokines, either on their own or in combination, did
not recruit p300 and p/CAF to the CXCL10 promoter. How-
ever, TNFα and IFNγ in combination induced a 5.5 ± 1.2-fold
increase in CBP binding to the CXCL10 promoter (Fig. 6C).

We also report that TNFα and IFNγ in combination recruit
RNA polymerase II to the CXCL10 promoter, allowing height-
ened transcription of the CXCL10 gene. TNFα and IFNγ alone
had a small effect on recruitment, inducing a 2.26 ± 0.18-fold
and 2.39 ± 0.29-fold increase, respectively. However, when
both cytokines were added in combination, a 5.85 ± 0.29-fold
increase in RNA polymerase II at the CXCL10 promoter was
achieved (raw values of immunoprecipitated RNA polymerase
II/input were as follows: control, 0.014 ± 0.003; TNFα, 0.029 ±
0.005; IFNγ, 0.29 ± 0.004; TNFα and IFNγ, 0.072 ± 0.013;
Fig. 6D).
and the selective IKK2 inhibitor ML120B (43). However, in contrast to previous reports (6), IFNγ neither induced NF-κB activation, nor was the release of CXCL10 induced by IFNγ in HASM cells grown on six-well plates were growth-arrested and treated with IFNγ (100 ng/ml) for 0–240 min. Protein was extracted and separated on a 10% SDS-PAGE gel and probed with an anti-human pJAK1, pJAK2, pSTAT-1α, or STAT-1α antibody. HT-29 cells stimulated with IL-4 were used as a positive control. Blots are representative of three independent experiments with similar results. C, confluent and serum-starved HASM cells in 75-cm² dishes were incubated with TNFα (1 ng/ml), IFNγ (100 ng/ml), or both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) for 1 h. The in vivo protein-DNA complexes were cross-linked by formaldehyde treatment, and chromatin pellets were extracted and sonicated. STAT-1α was immunoprecipitated with a specific antibody, and the associated CXCL10 promoter DNA was amplified by real-time PCR. The input represents PCR products from chromatin pellets prior to immunoprecipitation and is used to normalize the data. An IgG control (IgG) sample also was used to determine nonspecific background levels. Statistical analysis was performed on the absolute data using a t test with a Mann-Whitney posttest for single comparisons and a Kruskal-Wallis test with a Dunn’s post-test for multiple comparisons. *, significant (p < 0.05) difference from nonstimulated control group; †, significant (p < 0.05) difference from stimulated control group (mean ± S.E., n = 3).

DMSO, dimethyl sulfoxide.

FIGURE 4. Effect of inhibition of JAK on IFNγ-induced CXCL10 release and cytokines on STAT-1α phosphorylation and DNA binding. A, HASM cells grown on 96-well plates were growth arrested for 24 h and pretreated for 30 min with JAK inhibitor 1 (10⁻¹⁰ to 10⁻⁶ M) before stimulation with IFNγ (100 ng/ml) (A) or TNFα plus IFNγ (B) for 24 h. CXCL10 released into the supernatant was measured by ELISA, mean ± S.E. (n = 4 from independent donors). B, HASM cells grown on six-well plates were growth-arrested and treated with IFNγ (100 ng/ml) for 0–240 min. Protein was extracted and separated on a 10% SDS-PAGE gel and probed with an anti-human pJAK1, pJAK2, pSTAT-1α, or STAT-1α antibody. HT-29 cells stimulated with IL-4 were used as a positive control. Blots are representative of three independent experiments with similar results. C, confluent and serum-starved HASM cells in 75-cm² dishes were incubated with TNFα (1 ng/ml), IFNγ (100 ng/ml), or both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) for 1 h. The in vivo protein-DNA complexes were cross-linked by formaldehyde treatment, and chromatin pellets were extracted and sonicated. STAT-1α was immunoprecipitated with a specific antibody, and the associated CXCL10 promoter DNA was amplified by real-time PCR. The input represents PCR products from chromatin pellets prior to immunoprecipitation and is used to normalize the data. An IgG control (IgG) sample also was used to determine nonspecific background levels. Statistical analysis was performed on the absolute data using a t test with a Mann-Whitney posttest for single comparisons and a Kruskal-Wallis test with a Dunn’s post-test for multiple comparisons. *, significant (p < 0.05) differences from nonstimulated control group; †, significant (p < 0.05) difference from stimulated control group (mean ± S.E., n = 3).

TNFα and IFNγ Recruit CBP to the CXCL10 Promoter

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A

B

C

D

FIGURE 5. Identification of promoter cis-elements in cytokine-induced CXCL10 gene transcription. A, a full-length promoter used in these studies (−972). 50–60% confluent HASM cells in 24-well plates were cotransfected with the internal control Renilla luciferase (Luc) reporter plasmids (0.8 ng/well) and the CXCL10 promoter firefly reporter plasmids (0.4 μg/well) depicted in the figure for 18 h using FuGENE 6 transfection reagent and then treated with or without TNFα (61 ng/ml), IFNγ (C100 ng/ml), and both cytokines in combination (D0.1 ng/ml TNFα and 10 ng/ml IFNγ) for 6 h. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against the internal control Renilla luciferase activity. Statistical analysis was performed on the absolute data using a t-test with a Mann-Whitney post-test for single comparisons and a Kruskal-Wallis test with Dunn’s post-test for multiple comparisons. *, significant (p < 0.05) differences from nonstimulated control group; **, significant (p < 0.05) difference from stimulated control group (mean ± S.E., n = 3). ISRE, interferon-stimulated response element.

cells. We have previously used these cells to characterize the transcription factors and epigenetic events regulating the production of other chemokines and growth factors in airway and pulmonary vascular smooth muscle cells (24–25, 48–50).

In this study, we initially determined whether TNFα and IFNγ were able to synergistically enhance CXCL10 release via the recruitment of p65 or STAT-1α to the CXCL10 promoter and then further determined whether these cytokines in combination altered histone H3 and/or H4 acetylation. IFNγ has been reported to synergize with TNFα to augment expression of CD38 (51) and several chemokines, including CCL5, CXCL10, and CXCL11 (6, 26–27). Most studies that used a combination of IFNγ and TNFα showed that the synergistic action involves several molecular mechanisms. In some instances, their cooperatively may be explained by the IFNγ-induced up-regulation of TNFα receptors (52) or vice versa (53). Furthermore, both cytokines may collaborate at the gene level by increasing promoter activation through a synergistic interaction between transcription factors activated by IFNγ (STATs, IFN regulatory factor-1) and TNFα (NF-κB) (54–55). In the current experiments, the submaximal concentrations used were able to enhance synergistically CXCL10 release but have no effect on the activation of transcription factor binding; thus, we believe this mechanism does not explain our findings. Another mechanism underlying such cooperation could be the induction of defined genes including RANTES as well as CD38 by TNFα via activation of the autocrine action of IFNβ (39, 51). Although we saw no synergistic recruitment of p65, STAT-1α, or histone H4 when TNFα and IFNγ were used in combination, we did observe a synergistic recruitment of the coactivator CBP and RNA polymerase II to the CXCL10 promoter, heightening CXCL10 inflammatory gene regulation. With respect to the steroid resistance observed under these conditions, it is not known whether or not regulation of CXCL10 by transcription factors or cofactor recruitment to the CXCL10 promoter confer steroid insensitivity. Further work is needed to pinpoint the precise mechanism of steroid resistance.

Although we and others (6) report a synergistic induction of CXCL10 after stimulation of HASM cells with TNFα and IFNγ, it has been reported that IFNγ can inhibit TNFα-induced NF-κB reporter activity and mediator release (56). The molecular mechanisms are unclear but may involve the recruitment of other transcription factors in addition to effects on coactivators such as CBP in this study and/or additional cofactors as yet undefined.

This is the first study in HASM cells reporting the synergistic regulation of a coactivator and RNA polymerase II by inflammatory cytokines. Previously, Lee and colleagues (57) reported that in A549 cells, IL-1β recruits RNA polymerase II, along with p65 and c-Jun to the TGFβ1 promoter, in addition to histone H4 and H3 acetylation. Wada and colleagues (58) also reported that IL-1β enhanced recruitment of RNA polymerase II to the secretory leukocyte protease inhibitor gene. Additionally, Hiroi and colleagues (59) report similar findings to our results, in that the STAT-1/NF-κB-dependent transcriptional synergy observed after TNFα and IFNγ stimulation on CXCL9 release in mouse NIH3T3 and human embryonic kidney 293 cells could result from the enhanced recruitment of RNA polymerase II complex to the CXCL9 promoter via simultaneous interaction of CBP with STAT-1 and NF-κB.

Although steroid resistance is observed in only 5–10% of the asthmatic population, this leads to morbidity and mortality, and these subjects account for >50% of the United Kingdom healthcare costs for asthma (>2 billion pounds/annum; 60).
Additionally, steroids are relatively ineffective in acute exacerbations of asthma induced by virus (1, 2), where CXCL10 is increased (8). Alternative therapeutic interventions are therefore needed to address this unmet clinical need.

Inhibition of JAK3 has been reported to be an effective target in a murine collagen-induced and rat adjuvant-induced model of rheumatoid arthritis (61) and can drastically improve allograft survival post kidney transplantation (62). More recently, inhibition of this kinase has proven to be an effective anti-inflammatory therapy in a murine model of allergic pulmonary inflammation (63); therefore, clearly, these isoforms are tractable and can be developed for inflammatory diseases. Our data suggest that abrogation of JAK2 and subsequent STAT-1 signaling is more effective than the steroid fluticasone in an in vitro model of steroid-resistant inflammation and may provide a point of intervention in diseases such as severe asthma and COPD that are refractory to such treatment.

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FIGURE 6. Effect of cytokines on the in vivo acetylation of H4 and phosphorylation of RNA polymerase II (pol II) at the CXCL10 promoter. A–D, confluent and serum-starved HASM cells in 75-cm² dishes were incubated with TNFα (1 ng/ml), IFNγ (100 ng/ml), or both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) for 1 h. The in vivo protein-DNA complexes were cross-linked by formaldehyde treatment, and chromatin pellets were extracted and sonicated. Acetylated histone H4 (A), histone H3 (B), CBP (C), and RNA polymerase II (D) were immunoprecipitated with a specific antibody, and the associated CXCL10 promoter DNA was amplified by real-time PCR as described under “Experimental Procedures.” The input represents PCR products from chromatin pellets prior to immunoprecipitation and is used to normalize the data levels. IgG binding was unaltered after cytokine stimulation indicating specificity of antibodies uAn IgG (IgG) control sample also was used to determine nonspecific background. Data are mean ± S.E. of three separate experiments. E, HASM cells grown on 24-well plates were growth-arrested and treated with TNFα (1 ng/ml), IFNγ (100 ng/ml), or both cytokines in combination (0.1 ng/ml TNFα and 10 ng/ml IFNγ) in the absence and presence of the RNA polymerase II inhibitor α-amanatin for 24 h. CXCL10 released into the supernatant was measured by ELISA. Statistical analysis was performed on the absolute data with a t test using a Mann-Whitney post-test for single comparisons and a Kruskal-Wallis test with a Dunn’s post-test for multiple comparisons. To test for synergy between TNFα and IFNγ, the sum of TNFα (0.1 ng/ml) and IFNγ (10 ng/ml) alone was compared with TNFα and IFNγ and a Wilcoxon signed rank test was used. *, significant (p < 0.05) differences from nonstimulated control group; #, significant (p < 0.05) difference from stimulated control group (mean ± S.E., n = 3–4); #, significant (p < 0.05) difference between TNFα and IFNγ alone compared with the sum of TNFα and IFNγ.
