

CXCL8 Histone H3 acetylation is dysfunctional in airway smooth muscle in asthma: regulation
by BET

Rachel L. Clifford^a, Jamie K. Patel^a, Alison E. John^a, Amanda L. Tatler^a, Lisa Mazengarb^a,
Christopher E. Brightling^b and Alan J. Knox^{a#}

^a Department of Respiratory Medicine and Nottingham Respiratory Research Unit, University of
Nottingham, Nottingham, UK

^b Institute for Lung Health, Department of Infection, Immunity and Inflammation, University of
Leicester, Leicester, UK

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RLC, AJK.

Corresponding Author: Professor Alan J. Knox. Department of Respiratory Medicine and
Nottingham Respiratory Research Unit, University of Nottingham, Nottingham, UK

Tel: 0044 115 8231219. Fax: 0044 115 8231946. E-mail: alan.knox@nottingham.ac.uk

Abstract

Asthma is characterised by airway inflammation and remodelling and CXCL8 is a CXC chemokine that drives steroid resistant neutrophilic airway inflammation. We have shown that airway smooth muscle (ASM) cells isolated from asthmatic individuals secrete more CXCL8 than cells from non-asthmatic individuals. Here we investigated chromatin modifications at the CXCL8 promoter in ASM cells from non-asthmatic and asthmatic donors to further understand how CXCL8 is dysregulated in asthma. ASM cells from asthmatic donors had increased histone H3 acetylation, specifically histone H3K18 acetylation, and increased binding of histone acetyltransferase (HAT) p300 compared to non-asthmatics but no differences in CXCL8 DNA methylation. The acetylation reader proteins Brd3 and Brd4 were bound to the CXCL8 promoter and Brd inhibitors inhibited CXCL8 secretion from ASM cells by disrupting Brd4 and RNA polymerase II binding to the CXCL8 promoter. Our results show a novel dysregulation of CXCL8 transcriptional regulation in asthma characterised by a promoter complex that is abnormal in ASM cells isolated from asthmatic donors and can be modulated by Brd inhibitors. Brd inhibitors may provide a new therapeutic strategy for steroid resistant inflammation.

Introduction

Asthma is a chronic disease characterised by inflammation and remodelling of the airways. Clinical manifestations include repeated episodes of shortness of breath and wheezing, recurrent cough and excess mucus production. Airway smooth muscle (ASM) cells are a primary contributor to asthma pathogenesis. Traditionally considered as purely a contractile cell, ASM cells, contribute to airway inflammation and remodelling through the production of lipid mediators, chemokines (29), cytokines (47), growth factors (47), matrix metalloproteinases (17) and pro-angiogenic factors (11).

CXCL8 is a chemokine that mediates its effects via two G protein coupled receptors, CXCR1 and CXCR2. It is implicated in the pathogenesis of a variety of inflammatory and malignant diseases including inflammatory bowel disease (24), rheumatoid arthritis (22) non-small cell lung cancer (53) and asthma. In asthma, CXCL8 is a highly potent neutrophil chemoattractant causing their recruitment into the airways (50). Levels of CXCL8 are 19 times higher in tracheal aspirates from asthmatic subjects compared to non-asthmatic subjects and levels correlate with neutrophil number (42). Furthermore asthmatic individuals have higher sputum, plasma and exhaled breath condensate CXCL8 concentrations compared to healthy controls (12, 46). A higher proportion of airway epithelial cells express CXCL8 in asthmatic individuals compared to non-asthmatics (45) and we have shown that ASM cells isolated from asthmatic individuals express more CXCL8 protein and mRNA than those isolated from non-asthmatic controls (29). Identifying new mechanisms of CXCL8 modulation may therefore provide targets for treating severe asthma and other inflammatory diseases.

We showed previously that CXCL8 hypersecretion from ASM cells isolated from asthmatic donors was associated with increased binding of transcription factors and RNA polymerase II to the “asthmatic” CXCL8 promoter, but without an associated increase in transcription factor levels (29). Here we hypothesized that epigenetic modifications of the DNA and chromatin environment may underlie dysregulated CXCL8 transcription; specifically changes in either histone modifications or DNA methylation.

Histone acetyltransferases (HATs) catalyse the addition of acetyl groups to lysine residues present in core histone proteins, while histone deacetylases (HDACs) remove acetyl groups (31). Acetylated lysine residues are recognised by bromodomains, which exist in 46 diverse nuclear and cytoplasmic proteins including the bromodomain and extra-terminal (BET) proteins (20). The mammalian BET protein group includes Brd2, Brd3 and Brd4. Inhibitors of BET proteins selectively interfere with gene expression programmes that mediate cell growth, apoptosis and inflammatory responses (14, 15, 40).

A further degree of complexity is added to epigenetic transcriptional regulation by DNA methylation (5-mC), a reversible modification of cytosine residues mainly where a cytosine is followed directly by a guanine (CpG sites) (52). CpG methylation regulates gene expression via complex mechanisms including via direct inhibition of transcription factor binding and recruitment of methyl-binding domain (MBD) containing chromatin remodelling complexes.

Here we assessed chromatin modifications and DNA methylation levels at the CXCL8 promoter in ASM cells isolated from individuals with and without asthma and explored the potential for BET protein inhibitors as modulators of aberrant CXCL8 expression. We found that histone H3

acetylation, specifically histone H3 lysine 18 acetylation (H3K18Ac), was increased at the CXCL8 promoter in ASM cells from asthmatic patients. Increased H3K18Ac was associated with increased recruitment of the histone acetyltransferase p300. In contrast DNA methylation levels were similar in ASM cells from asthmatic and non-asthmatic donors. Finally we found that CXCL8 expression can be modulated via BET protein inhibition of BRD4 and RNA polymerase II association with the CXCL8 promoter.

Materials and Methods

Cell culture

Primary cultures of HASM cells were isolated from bronchial biopsies and large airway tissue from subjects undergoing surgery at Glenfield Hospital, Leicester and cultured as previously described (11, 29). The isolation and culture techniques were identical for asthmatic and non-asthmatic donors. Asthmatic subjects and non-asthmatic controls from which cells were isolated were recruited from Leicester, UK. Subjects with asthma had a consistent history and objective evidence of asthma, as indicated by one or more of the following: (i) methacholine airway hyperresponsiveness (AHR) [PC_{20} forced expiratory volume in 1s (FEV_1) <8 mg/mL]; (ii) >15% improvement in FEV_1 15 min after administration of 200 µg of inhaled salbutamol or (iii) >20% of maximum within-day amplitude from twice daily peak expiratory flow measurements over 14 days. Severity of asthma was defined by Global Initiative for Asthma (GINA) guidelines (33). The study was approved by the Leicestershire Research Ethics Committees and all patients gave their written informed consent (30). Four non-asthmatic individuals were non-smokers and two were ex-smokers. Four of the asthmatic individuals were non-smokers and three were ex-smokers. To maintain statistical power we did not distinguish between non and ex-smokers in our observations. Cells were cultured in DMEM (Sigma) containing 10% fetal bovine serum (Gibco, Life Technologies, UK), penicillin (100U/ml), streptomycin (100µg/ml) and L-glutamine (4mM) in a humidified 5% CO₂ humidified incubator. Experiments were performed at passage 5 or 6. A minimum of three non-asthmatic lines and three asthmatic primary cultures were used per experiment. Cells were growth arrested 24 hours prior to experiment initiation. For studies in which the effect of inhibitors was assessed by measuring supernatant CXCL8 protein concentrations by ELISA, cells were cultured in 48 well plates. For real time analysis of CXCL8 mRNA expression, HASM cells were cultured in 6 well plates. For isolation of genomic DNA,

cells were cultured in T75 flasks and for Chromatin Immunoprecipitation (ChIP) assays, cells were cultured in T225-cm² flasks.

BET inhibitor studies

Concentration response. Media were replaced with serum-free media containing 0.1% DMSO, 0.1µM/1µM/10µM PFI-1 (Tocris Bioscience, Bristol, UK), 0.1µM/1µM/10µM I-BET (Tocris) or 0.01µM /0.1µM/1µM JQ-1 (Cayman Chemical, Michigan, USA) compounds. Supernatants were collected 24 hours later and assayed by ELISA.

Human CXCL8 ELISA

The human CXCL8 ELISA (R&D systems) was performed according to the manufacturer's protocols. CXCL8 concentrations were normalised to cell counts and expressed relative to the mean CXCL8 concentration in DMSO treated samples in cells from non-asthmatic donors. To compare the extent of inhibition between cells from asthmatic and non-asthmatic donors we also expressed data relative to each donor cell's DMSO control.

RNA expression. Media were replaced with serum-free media containing 0.1% DMSO, 10µM PFI-1, 1µM I-BET or 1µM JQ-1 compounds. 2 hours later cells were lysed for RNA extraction.

RNA isolation and RT-PCR

Total RNA was isolated, reverse transcribed and subjected to real time PCR as described previously (11). Human CXCL8 primers sequences were: forward 5'-

ATGACTTCCAAGCTGGCCGTGGCT-3' and reverse 5'-

TCTCAGCCCTCTTCAAAACTTCTC-3'. β₂-microglobulin was used as a housekeeping gene

(forward 5'- AATCCAAATGCGGCATCT-3', reverse 5'- GAGTATGCCTGCCGTGTG-3').

Expression was expressed using the $\Delta\Delta\text{Ct}$ method relative to $\beta_2\text{M}$ Ct and within donor cell line

DMSO control CXCL8/ $\beta_2\text{M}$ ΔCt .

Cell Viability

The toxicity of all chemicals and vehicles was determined by MTT assay. At the end of the experiment culture media were removed and replaced with 250 μl of serum-free media containing 1 mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) (Sigma), and then incubated for 20 min at 37 °C. This medium was removed, and the plates were dried overnight. 250 μl of dimethyl sulfoxide (DMSO) was then added to dissolve the blue-colored tetrazolium. The absorbance was read at 550 nm in a FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). Viability was set as 100% in control cells.

Pyrosequencing

DNA was extracted using the QIAamp DNA Mini kit (Qiagen). 2 μg of genomic DNA was bisulfite converted using the EpiTect Bisulfite kit (Qiagen). Eluted DNA was used as a PCR template. Primers for PCR amplification and sequencing were designed (PyroMark[®] Assay Design 2.0 software (Qiagen)) and are given in Table I and indicated in Figure 2A. PCRs were performed with the HotStarTaq DNA polymerase PCR kit (Qiagen) under the following conditions: 95°C 5 minutes; 45 cycles of 94°C 30 seconds; 56°C 30 seconds; 72°C 30 seconds; finally 72°C 10 minutes. Except the PCR primers for CpGs 7 & 8 which required an annealing temperature of 52.6°C. Amplification success was assessed by agarose gel electrophoresis and the resulting products pyrosequenced with the Pyromark[™] Q24 System (Qiagen).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using the ChIP-IT Express kit (Active Motif) following the manufacturers protocol. Chromatin was fragmented using the Q800R Sonicator® (Active Motif). Antibody amounts and product codes are stated in Table II. Input and IgG/whole serum controls were performed in parallel. Input DNA was phenol/chloroform extracted before being used in PCR. Products were amplified by quantitative real-time PCR as described previously (29). Association is expressed using the $\Delta\Delta\text{Ct}$ method relative to Input Ct and either the mean non-asthmatic input/IP ΔCt or within donor cell line DMSO control input/IP ΔCt for I-BET inhibitor studies.

Statistical Analysis

Data are expressed as means $\pm$ SEM of n determinants. N is stated in figure legends. N refers to the number of cell donors used per experiment. Statistical analyses were performed using GraphPad Prism Software (Version 6). Unpaired two-tailed Student t -tests were used to determine significant differences, a $p < 0.05$ was considered significant.

Results

Increased CXCL8 expression from ASM cells from asthmatic individuals is associated with altered histone acetylation. We first investigated differences in histone modifications at the CXCL8 promoter in ASM cells from non-asthmatic versus asthmatic individuals. As the CXCL8 promoter has been shown previously to be regulated by histone acetylation and methylation of H3 lysine 4 and H3 lysine 9 (5) we measured the levels of these modifications at the CXCL8 promoter using chromatin immunoprecipitation (ChIP) and primers that amplify the region -121 to +67 bp relative to the transcription start site (29). While histone acetylation is generally associated with active transcription, the role of histone methylation is more complex and depends on the position and extent (mono, bi, tri) of the methylation. Di-, or trimethylation of lysine 9 on histone H3 (H3K9me2/3) is associated with transcription repression and heterochromatin formation (34), while tri- and dimethylation of lysine 4 on histone H3 (H3K4me2/3) are found at actively transcribing genes (34). While we saw a reduced level of H3K9me3 associated with the CXCL8 promoter in cells isolated from asthmatic individuals compared to those from non-asthmatic individuals (Fig 1A) and an increased association of H3K4me3 with the CXCL8 promoter in ASM cells isolated from asthmatic individuals (Fig 1B), neither reached statistical significance and were not investigated further. Subsequently we investigated histone H3 and H4 acetylation. Interestingly while there was no difference in the levels of histone H4 acetylation at the CXCL8 promoter in cells from asthmatic individuals compared to non-asthmatic individuals (Fig 1C), histone H3 acetylation at the CXCL8 promoter was increased in cells from asthmatic individuals (Fig 1D) and we went on to investigate this further.

ASM cell CXCL8 hypersecretion from asthmatic individuals is not associated with differences in CXCL8 DNA methylation. The CXCL8 gene sequence contains 8 CpG sites within the region

1500bp upstream and 150bp downstream of the transcription start site (Figure 2A). We assessed the percent methylation at each CpG site in ASM cells isolated from asthmatic individuals and non-asthmatic controls using quantitative Pyrosequencing. CpG site methylation varied across the DNA (Figure 2B). Methylation was highest at the CpGs located distally upstream of the transcription start site and decreased to a minimum level at CpG8 located downstream of the transcription start site. However we observed no difference in CXCL8 CpG methylation between cells isolated from asthmatic individuals versus non-asthmatic individuals, suggesting that DNA methylation does not play a role CXCL8 hypersecretion from asthmatic ASM cells.

Increased acetylation at the asthmatic CXCL8 promoter is specific to histone H3 lysine 18 acetylation and is associated with increased histone acetyltransferase binding. To understand in greater the detail the histone H3 acetylation landscape at the CXCL8 promoter in ASM cells from asthmatic and non-asthmatic donors we performed ChIP using antibodies for four specific histone acetylation sites, lysine 9 (H3K9Ac), lysine 14 (H3K14Ac), lysine 27 (H3K27Ac) and lysine 18 (H3K18Ac). While we found no differential in acetylation between the asthmatic and non-asthmatic cells at sites K9, K14 and K27 (Fig 3 A-C) we found that H3K18Ac was barely detectable over the IgG control in ASM cells from non-asthmatic donors but was increased in the cells isolated from asthmatic individuals (Figure 3D).

To understand why H3K18Ac was increased we investigated the binding of histone acetyltransferases (HATs), the enzymes responsible for depositing acetyl groups on histone tail lysine residues, to CXCL8 promoter. There are approximately 30 known HATs in humans that are grouped into 5 families based on the structural and functional similarity of their catalytic domains. Here we focused on the HATs p300 (28) and P300/CBP-associated factor (PCAF) (1)

as they are known to acetylate H3K18. As with H3K18Ac we observed little signal for either p300 (Figure 3E) or PCAF (Figure 3F) over the IgG control in non-asthmatic ASM cells but observed increased binding in asthmatic ASM cells, with p300 reaching statistical significance. This suggests that aberrant H3K18Ac at the CXCL8 promoter in ASM cells from asthmatic donors is caused by inappropriate recruitment of HATs to the CXCL8 promoter.

The histone acetylation reader proteins, Brd3 and Brd4, are present at the CXCL8 promoter and BET inhibitors reduce CXCL8 secretion from ASM cells.

Bromodomain (BRD) containing proteins are a class of histone modification reader proteins that specifically recognise acetylated lysine residues. They play crucial roles in transcriptional regulation by acting as scaffolds for macromolecular complexes that alter chromatin accessibility to transcription factors and RNA polymerases. The BET proteins, Brd2, Brd3, Brd4 and BrdT are one family of BRD containing proteins. Importantly the first selective nanomolar inhibitors for these proteins have been identified and have shown translatable promise in models of midline carcinoma (21) and lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis (40). This is in contrast to direct inhibitors of histone acetylation which have struggled in drug development due to lack of selectively and pleotropic effects. Due to the fact that histone acetylation is present and dysregulated at the CXCL8 promoter we aimed to identify whether BET family proteins were bound to the CXCL8 promoter. We performed ChIP for Brd2, 3 and 4. BrdT was not investigated as it has testis specific expression. ChIP for BRD2 showed no enrichment for BRD2 binding over IgG control (Figure 4A) suggesting BRD2 is not associated with the CXCL8 promoter. However both BRD3 (Figure 4B) and BRD4 (Figure 4C) were associated with the CXCL8 promoter (significant difference between target IP and IgG). No differences in BRD

protein binding were seen between cells isolated from asthmatic donors and those from non-asthmatic donors.

To determine whether BET proteins were regulating CXCL8 expression we measured the effect of three different BET protein inhibitors on CXCL8 protein secretion and mRNA expression in ASM cells from both asthmatic and non-asthmatic donors. Three structurally different compounds we used were: the potent and highly selective dihydroquinazoline-2-one inhibitor, PFI-1, (44), I-BET (40) and the thienodiazepime JQ1. The enantiomerically pure (+)- JQ1 inhibits BET proteins while the (-)- JQ1 stereoisomer has no effect and can be used as a negative control compound (21). Here cells were serum starved for 24 hours, the media replaced with fresh media containing the stated concentration of compound and supernatants and RNA samples collected at 24 hours and 2 hours respectively. As shown previously ASM cells from asthmatic donors secreted significantly more CXCL8 than those from non-asthmatic donors (data shown is percent CXCL8 relative to the mean CXCL8 levels of non-asthmatic DMSO samples). PFI-1 (Figure 5A), I-BET (Figure 5B) and JQ1+/- (Figure 5C) significantly reduced CXCL8 protein secretion into the cell culture supernatant. The negative control compound JQ1-/- had no effect on CXCL8 secretion (Figure 5D). When data were expressed as a percentage of each donor cell's DMSO control (Figure 6) all compounds showed a similar extent of inhibition at the highest concentration used, with the percent CXCL8 remaining ranging from 41.1% (10 μ M PFI-1 with non-asthmatic cells) to 54.3% (10 μ M I-BET with asthmatic cells). There was no difference in the extent of inhibition between ASM cells isolated from asthmatic donors versus those from non-asthmatic donors. To ensure that inhibition was not accounted for by cell toxicity MTT assays were performed. As shown in Figure 7 none of the inhibitors or the JQ-/- control compound caused any cell toxicity. CXCL8 mRNA expression (Figure 5E) was significantly inhibited by

10 μ M PFI-1 and 1 μ M I-BET in ASM cells from both asthmatic and non-asthmatic individuals. 1 μ M JQ1+/- showed a decrease in CXCL8 mRNA levels compared to 1 μ M JQ1-/- in both ASM cells from asthmatic and non-asthmatic donors. These data suggest that BET proteins are involved in the transcriptional regulation of CXCL8. This may offer a novel, indirect and potentially translatable approach to modulating aberrant histone acetylation dependent gene expression in asthma, by targeting the reader BET proteins rather than the writer HATs directly.

BET protein inhibitors mediate their action via disruption of BRD4 and RNA polymerase II, but not transcription factor, association with the CXCL8 promoter. Finally we wanted to establish the molecular mechanism of CXCL8 inhibition by BET inhibitors. The three BET inhibitors used are known to act by mimicking acetylated histones and preventing Brd protein chromatin association. As such we performed ChIP for Brd4 and Brd3 on chromatin from cells cultured with or without the BET inhibitors PFI-1 and I-BET (10 μ M and 1 μ M respectively). Preliminary single cell line time course experiments (data not shown) showed that BRD3 binding was not affected by either 1 μ M I-BET or 10 μ M PFI-1 and that BRD4 binding to the CXCL8 promoter was more efficiently inhibited by I-BET than PFI-1. BRD4 dissociation had occurred within 30 minutes of BET protein inhibitor incubation (data not shown). A subsequent ChIP experiment showed that I-BET significantly inhibited BRD4 binding to the CXCL8 promoter in ASM cells from asthmatic donors and reduced binding in cells from non-asthmatic donors (Figure 8A). We have shown previously that NF- κ B p65 is present and active in the nucleus of unstimulated ASM from asthmatic and non-asthmatic individuals and that hypersecretion of CXCL8 from ASM cells from asthmatic donors is associated with increased binding of the transcription factors NF- κ B p65 and C/EBP β (29). Inhibition of hydrogen peroxide and interleukin 1 β stimulated CXCL8 secretion from immortalized human bronchial epithelial cells

by JQ1+/- is mediated via inhibition of p65 binding to the CXCL8 promoter (31). Hence we investigated whether in the current context the action of I-BET was mediated via modulation of p65 and C/EBP β binding to the CXCL8 promoter. As shown in Figures 8B and 8C respectively, while p65 and C/EBP β are present at the CXCL8 promoter, I-BET had no effect on p65 binding and little effect on C/EBP β binding. Finally, as RNA polymerase II (Pol II) is required for mRNA transcription and we have previously shown increased Pol II association with the CXCL8 promoter in asthmatic ASM cells we investigated the effect of BET protein inhibition on Pol II association. Figure 8D shows that 1 μ M I-BET significantly reduced the levels of RNA Pol II associated with the CXCL8 promoter in ASM cells from both asthmatic and non-asthmatic individuals. These data suggest that BET protein inhibitors mediate their effects at the CXCL8 promoter in ASM cells via disruption of BRD4 and Poll II while leaving the measured transcription factor complex in place.

Discussion

The main finding in this study is that histone acetylation is dysregulated at the CXCL8 promoter in ASM cells from asthmatic donors. Furthermore we show that the histone acetylation binding BET proteins are critical to CXCL8 expression and that BET protein inhibitors offer a novel therapeutic option for modulating CXCL8 in asthma and potentially other neutrophilic diseases.

We chose to study CXCL8 because it is a key chemoattractant for neutrophils and neutrophilic inflammation is a feature of steroid-resistant asthma, which is difficult to treat. Studies in humans with asthma and mouse asthma models show that CXCL8 blocking strategies are beneficial. The selective CXCR2 receptor antagonist SCH527123 reduced sputum neutrophil counts and mild exacerbations in severe asthma patients (39). Furthermore blockade of the murine CXCL8 receptors CXCR1 and CXCR2, reduced neutrophilic airway inflammation in mouse models of various pulmonary conditions including asthma (23). We studied CXCL8 expression regulation in cultured ASM cells from asthmatic and non-asthmatic donors as we have previously shown that cells from asthmatic donors hypersecrete CXCL8 (29). This change in expression is stable with repeated passage in culture suggesting a fundamental change in cell phenotype. Furthermore changes in cytokine expression are seen in intact human asthma tissue (45).

We found the most striking observation at the CXCL8 promoter in cells from asthmatic donors was a large increase in acetylated histone H3. We previously described dysregulated histone lysine methylation regulating aberrant VEGF expression in ASM cells from asthmatic patients (11). Specifically a lack of the repressive H3K9me3 mark and an increase in the active H3K4me3 mark was observed at the VEGF promoter in cells from asthmatic donors. While we

did see some reduction in H3K9me3 and some increase in H3K4me3 at the CXCL8 promoter in ASM cells from asthmatic donors these histone modifications were not statistically significant. Similarly in our previous studies of the VEGF promoter no changes in histone H3 acetylation were seen. These data suggest that where there is a lack of difference between our cells from asthmatic and non-asthmatic donors it is due to different underlying molecular mechanisms at the promoters rather than technical experiential issues. Acetylation regulates gene expression via two primary methods; alteration of the nucleosome confirmation from a closed to an open state allowing access for the transcriptional machinery and the generation of binding motifs on the nucleosome surface for reader/effector proteins which assist in recruiting the molecular complexes required for active transcription (10). Previous studies have shown that CXCL8 expression is regulated by either histone H3 or H4 histone acetylation in several cell types and in response to a range of stimuli (3, 8) and we have shown that TNF α induction of CXCL8 expression in ASM cells is associated with histone H4 acetylation (41). However to our knowledge regulation of CXCL8 by histone H3 acetylation has not previously been reported in ASM cells. Furthermore while most previous publications have measured pan histone acetylation we investigated 4 specific histone H3 acetylation sites and identified dysfunction in a single site, H3K18. Few studies have aimed to identify the individual roles of specific histone lysine residue acetylation. Those studies that have are mainly in cancers. In *Saccharomyces cerevisiae* it was proposed that lysines 4,9,14,18, 23 and 27 on histone H3 have redundant functions and act as a group to mark euchromatin, however they did not include mutation analysis of K18 alone in their study (37). H3K18 deacetylation is required for host responses to bacterial infection (18) and H3K18 hypoacetylation is linked to maintenance of a malignant phenotype (7) and poor prognosis in prostate cancer (49) and pancreatic adenocarcinoma (36) suggesting that acetylation of lysine 18 has a specific cellular role. It would be of interest in future studies to assess whether

increased H3K18Ac is more widespread in ASM cells from asthma subjects (and whole lung tissue incorporating the many cell types involved in asthma pathogenesis) or whether it is specific to CXCL8. The increased H3K18 acetylation was associated with a concomitant increase in association of the acetylation writer protein, histone acetyltransferase (HAT) p300. We also observed a trend towards increased PCAF binding. Both p300 and PCAF can acetylate multiple histone lysine residues on both histone H3 and H4 (26, 48) and it is unclear at present what regulates the sites they acetylate under any given condition. Speculatively the presence of neighbouring or associated histone modifications may play a role. We attempted to assess this here but experienced technical barriers. As the lack of specificity and toxicity of available HAT inhibitors makes them unsuitable to unravel the molecular function of H3K18ac in whole cell systems we performed siRNA studies to knock down p300 and PCAF (data not shown). Unfortunately this approach induced cell toxicity likely reflecting the fundamental roles of these proteins in regulating diverse cellular functions including cell growth, differentiation and apoptosis.

It would be of further future interest to understand the mechanisms driving altered HAT association with the CXCL8 promoter. Changes in the epigenetic profiles of airway structural cells are likely initiated by insults from inhaled stimuli, including allergens, respiratory viruses and air pollutants. Little is known about the direct effect of allergens on epigenetic mechanisms including HAT recruitment. However in response to allergens, T helper (T_H) lymphocytes are differentiated into T_H2 cells and T_H2 differentiation is accompanied by alterations in the acetylation state of histones at cytokine gene loci including the allergic inflammatory cytokine interleukin-4 (19, 25) and interferon gamma (19) suggesting HAT activation occurs in response to allergen exposure(27). The major air pollutant, diesel exhaust, regulates cyclooxygenase 2

(COX-2) expression in airway epithelial cells via increased recruitment of p300 to the COX-2 promoter (9). While little is known about respiratory virus effects on HATs in the lung, viruses are well known to interact with HATs in cancer. The HPV 6, 16 and 18 E7 proteins of a high-risk human papillomavirus (HPV) involved in cervical cancer directly interact with PCAF (4) and the cellular protein AMF-1 (Gps2) positively modulates gene expression by the papillomavirus E2 protein via direct interaction with p300 (43). Taking this evidence into account we speculate that exposure to inhaled environmental exposures induces stable alterations in the recruitment of epigenetic writer proteins, included HATs, to specific DNA regions in airway structural cells including ASMs that affects specific gene expression, cell function and contributes to asthma pathogenesis.

In addition to assessing histone changes we investigated the DNA methylation status of CpG sites within the CXCL8 promoter. The majority of DNA methylation studies are performed at genes that contain large regions rich in CpG sites (CpG islands) as hypermethylation of cytosines within these regions silences gene transcription. Although the CXCL8 promoter does not contain a CpG island and is therefore an unlikely candidate for DNA methylation analysis, dysregulated methylation of individual CpG sites within the CXCL8 promoter has been described in non-small cell lung cancer (51) and colorectal adenocarcinoma (16) and associated with altered gene expression. We found no differences in CXCL8 promoter DNA methylation between ASM cells from asthmatic and non-asthmatic donors. Our study is however the first to quantitatively assess methylation of all 8 CpGs within the 1650bp region of the CXCL8 transcription start site. We found that there was a decrease in CpG methylation as proximity to the transcription start site was reduced consistent with a recent genome-wide study of DNA methylation in 17 somatic

tissues that showed decreasing methylation the closer to the TSS at non-CpG island DNA regions (35).

No highly specific HAT inhibitors have been developed and we postulated that targeting “reader” proteins might prove a better therapeutic strategy in our cells. “Reader” proteins recognise histone modification marks and either play a direct role in transcriptional regulation or recruit proteins/complexes that regulate transcription. In contrast to HATs, BET proteins have been shown to be targetable by selective inhibitors that have a good therapeutic profile. We found that 2 BET proteins, Brd3 and Brd4, were present at the CXCL8 promoter in ASM cells from both asthmatic and non-asthmatic donors and that three different BET protein inhibitors, PFI-1, I-BET and JQ(+/-) reduced CXCL8 protein secretion and mRNA levels. There were no differences in the levels of Brd3 or Brd4 recruitment to the CXCL8 promoter in cells from asthmatic versus non-asthmatic donors. This is not necessarily surprising as we observe very specific differential histone H3 lysine 18 acetylation at the asthmatic CXCL8 promoter and the Brd proteins are likely also associated with other acetylated residues at the CXCL8 promoter. What is important to the current study is that inhibition of these BET proteins normalised the levels of CXCL8 secretion from ASM cells from asthmatic patients to those secreted from ASM cells from non-asthmatic individuals. JQ(+/-) has previously been shown to inhibit hydrogen peroxide and interleukin 1 β induced CXCL8 secretion from immortalised airway epithelial cells (31) but ours is the first report of their efficacy in primary ASM cells and their ability to normalise aberrant cytokine secretion in cells from asthmatic donors. Our results differ from those of Khan *et al* (31) who found the BET inhibitor affect in immortalized airway epithelial cells to be mediated via disruption of Brd4 and p65 binding to the CXCL8 promoter whereas we observed no effect on p65 or CEBP transcription factor binding but rather a reduction in Brd4

and RNA polymerase II association with the CXCL8 promoter. Interestingly the Khan study also showed that while reduction of Brd4 protein levels mimicked the effects of the pharmacological IBET inhibitor, reduction of Brd2 protein levels had no such effect. Similarly in our study we show that IBET disrupts Brd4 association with the CXCL8 promoter but has no effect on Brd3 binding (data not shown). This suggests that these inhibitors may exhibit context specific effects. BET inhibitor compounds were not toxic to our primary ASM cells (MTT assays) adding to their potential as therapeutics.

Although our studies focussed on ASM CXCL8 production in asthma, the findings may have broader applicability to other diseases driven by neutrophilic inflammation. In rheumatoid arthritis (RA), a chronic inflammatory disease affecting the diarthrodial joints, CXCL8 mediates neutrophil recruitment (32) and BET protein inhibition reduces collagen induced arthritis in mice (38). BET inhibitors also protect against lipopolysaccharide-induced endotoxic shock, bacteria induced sepsis (40) and cause long lasting suppression of Th1 cell proinflammatory function (6), via regulation of inflammatory gene expression. Heart failure includes inflammatory pathway activation in its pathology (2) and plasma CXCL8 levels correlate with worsening chronic heart failure (13) and in an in vitro phenylephrine induced model of heart failure in neonatal rat ventricular cardiomyocytes BET protein inhibition reduced expression of pro-inflammatory genes (2). All of these studies highlight a strong involvement of BET proteins in inflammatory disease.

In summary our study strongly suggests that the chromatin/transcriptional complex at the unstimulated CXCL8 promoter is fundamentally different in ASM cells isolated from asthmatic individuals compared to non-asthmatic individuals. This is characterised by increased RNA

polymerase II, C/EBP β and p65 binding (previously published (29)) and aberrant presence of H3K18Ac and p300 at the asthmatic promoter (Figure 9). Furthermore CXCL8 transcription is dependent on the presence of histone acetylation reader proteins Brd3 and Brd4 and BET protein inhibitors can modulate CXCL8 expression via disruption of Brd4 and RNA polymerase II association with the promoter. BET protein inhibitors may have therapeutic potential against steroid-resistant neutrophilic asthma and other inflammatory diseases where neutrophilic inflammation is driven by CXCL8.

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723 **Table I. Pyrosequencing Primers**

CpG Site	PCR Forward Primer (5'-3')	PCR Reverse Primer (5'-3')	Sequencing Primer (5'-3')
1	TGTTTATAGTGTGGGTAA ATTTATTGT	ATCCTAAAAAAAAAAAAATC CAAAACCT (Bio)	TGGGTAAATTTATTGTTT TGT
2	“ “	“ “	ATAAATTATGTATTTGTT TAGAAG
3	GTGGAGTTTTAGTATTTT AAATGTATAT (Bio)	ATCACACTTCCTATTTATT CCTTATCA	ACTTCCTATTTATTCCTT ATCAA
4	“	“	“
5	TTGAGGGGATGGGTATT AGTT	ACTTATACACCCTCATCT TTTCAT(Bio)	GGATGGGTATTAGTTG TA
6	GTGTATAAGTTTTTTAGT AGGGTGATG	AATCAAAAAAACTACCA AAAAAACCT(Bio)	AGGGTGATGATATAAAA AGT
7	“ “	“ “	AGGATAAGAGTTAGGA AGA
8	“ “	“ “	ATTGTGTGTAAATATGA TTTTTAA

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Table II: ChIP antibodies and amounts used in IPs

Antibody Target	Manufacturer	Product code	Amount in IP
H3K9me3	Millipore	17-625	4µg
H4K4me3	Active Motif	39159	4µl (whole serum)
Pan H4Ac	Millipore	06-598	4µg
Pan H3Ac	Millipore	06-599	4µg
H3K9Ac	Active Motif	39137	10µl (whole serum)
H3K14Ac	Active Motif	39599	10µl (whole serum)
H3K27Ac	Active Motif	39135	4µl (whole serum)
H3K18Ac	Active Motif	39693	4µl (whole serum)
p300	Santa Cruz	Sc-9001X	5µg
P/CAF	Santa Cruz	Sc-6300 X	5µg
BRD2	Santa Cruz	Sc-46805	3µg
BRD3	Santa cruz	Sc-99192	3µg
BRD4	Bethyl Labs	A301-985A50	5µg
p65	Santa Cruz	Sc-372X	5µg
CEBP	Santa Cruz	Sc-150X	5µg
RNA polymerase II	Santa Cruz	Sc-9001	5µg

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Figure Legends

Figure 1. CXCL8 hypersecretion is associated with altered histone acetylation.

A) Histone H3 lysine 9 trimethylation (H3K9me3), B) Histone H3 lysine 4 trimethylation (H3K4me3), C) pan acetylated Histone H4 (H4Ac) and D) pan acetylated Histone H3 (H3Ac) association with the CXCL8 promoter was assessed by ChIP. Binding was measured in confluent, serum-deprived ASM cells from asthmatic and non-asthmatic donors. IgG negative controls are shown. Data are expressed as fold change relative to the mean non-asthmatic IP value. ** $p < 0.01$ comparing asthmatic with non-asthmatic. A), B) and D) $n = 3$ non-asthmatic and 3 asthmatic donors ASM lines. C) $n = 3$ non-asthmatic and 5 asthmatic donors ASM lines.

Figure 2. CXCL8 hypersecretion is not associated with differences in DNA methylation.

Genomic DNA was isolated from ASM cells from 3 asthmatic and 3 non-asthmatic individuals and bisulfite converted. Regions of DNA containing CpG sites were PCR amplified and pyrosequenced. A) Schematic showing the position of the 8 CXCL8 CpG sites, the PCR primers (blue arrows) and sequencing primers (orange arrows). B) The percent methylation of the individual CpG sites in ASM cells from both asthmatic and non-asthmatic individuals.

Figure 3. Increased CXCL8 promoter histone acetylation is specific to histone H3 lysine 18

acetylation and is associated with increased acetyltransferase binding. A) Histone H3 lysine

9 acetylation (H3K9Ac), B) Histone H3 lysine 14 acetylation (H3K14Ac), C) Histone H3 lysine

27 acetylation (H3K27Ac), D) Histone H3 lysine 18 acetylation (H3K18Ac), E) p300 and F)

PCAF association with the CXCL8 promoter was assessed by ChIP. Binding was measured in

confluent, serum-deprived ASM cells from asthmatic and non-asthmatic donors. IgG negative

controls are shown. Data are expressed as fold change relative to the mean non-asthmatic IP

value. $*p < 0.05$ comparing asthmatic with non-asthmatic. A), B), C), E) and F) $n = 3$ non-asthmatic and 3 asthmatic donors ASM lines. D) $n = 4$ non-asthmatic and 3 asthmatic donors ASM lines.

Figure 4. Brd3 and Brd4 are present at the CXCL8 promoter. A) Brd2, B) Brd3 and C) Brd4 association with the CXCL8 promoter was assessed by ChIP. Binding was measured in confluent, serum-deprived ASM cells from asthmatic and non-asthmatic donors. IgG negative controls are shown. Data are expressed as fold change relative to the mean non-asthmatic IP value. $*p < 0.05$ comparing target IP to IgG control. A) and B) $n = 5$ non-asthmatic and 5 asthmatic donors ASM lines. C) $n = 4$ non-asthmatic and 4 asthmatic donors ASM lines.

Figure 5. BET inhibitors reduce CXCL8 secretion and expression from ASM cells. CXCL8 protein levels following incubation with A) PFI-1, B) I-BET C) JQ+/- and D) JQ-/. Supernatants were collected from confluent, serum deprived ASM cells following 24 hour incubation with the stated concentration of compound. $**p < 0.01$, $****p < 0.0001$ comparing asthmatic with non-asthmatic DMSO control. $+p < 0.05$, $++p < 0.01$ compared with non-asthmatic DMSO control. $\#p < 0.05$, $##p < 0.01$ compared with asthmatic DMSO control. E) CXCL8 mRNA levels as determined by real time PCR following 2 hour incubation with 10 μ M PFI, 1 μ M I-BET, 1 μ M JQ+/- and 1 μ M JQ-/. Data are expressed relative to each donor lines DMSO control. β 2M was used as a housekeeping gene. $+++p < 0.001$, compared with non-asthmatic DMSO control. $####p < 0.001$, $#####p < 0.0001$ compared with asthmatic DMSO control. A) and B) $n = 3$ non-asthmatic and 3 asthmatic donors. C) and D) $n = 5$ non-asthmatic and 4 asthmatic donors. E) $n = 4$ non-asthmatic and 4 asthmatic donors.

Figure 6. There is no difference in the BET inhibitory effect between ASM cells from asthmatic and non-asthmatic donors. CXCL8 protein levels following incubation with A) PFI-1, B) I-BET C) JQ+/- and D) JQ-/- . Supernatants were collected from confluent, serum deprived ASM cells following 24 hour incubation with the stated concentration of compound. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, ++++ $p < 0.0001$ compared with non-asthmatic DMSO control. # $p < 0.05$, ## $p < 0.001$, ### $p < 0.001$, #### $p < 0.0001$ compared with asthmatic DMSO control. A) and B) $n = 3$ non-asthmatic and 3 asthmatic donors. C) and D) $n = 5$ non-asthmatic and 4 asthmatic donors

Figure 7. BET inhibitors are not toxic to ASM cells. Percent cell viability following incubation with A) PFI-1, B) I-BET C) JQ+/- and D) JQ-/- . 250 μ l 1mg/ml MTT solution was added to rinsed ASM cells following removal of the supernatants. Cells were incubated for 20 minutes at 37°C, the MTT removed and dried overnight. 250 μ l DMSO was used to re-suspend the MTT and the absorbance at 550nm read. Data are expressed as percentages relative to DMSO controls and are derived from the same n as matched experiments in Figures 5 A-D.

Figure 8. BET inhibitors mediate their affects via disruption of Brd4 and RNA Polymerase II association with the CXCL8 promoter. Changes in A) Brd4, B) p65, C) CEBP and D) RNA polymerase II association with the CXCL8 promoter following 30 minute incubation with 1 μ M I-BET with was assessed by ChIP. Binding was measured in confluent, serum-depreived ASM cells from asthmatic and non-asthmatic donors. IgG negative controls are shown. Data are expressed as fold change relative to within donor control IP value. ** $p < 0.01$, **** $p < 0.0001$ comparing IBET with DMSO control. A) and B) $n = 4$ non-asthmatic and 4 asthmatic donor ASM lines. C) and D) $n = 3$ non-asthmatic and 4 asthmatic donors ASM lines.

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809 **Figure 9. Summary Schematic.** Fundamental differences exist between the molecular
810 complexes at the CXCL8 promoter in ASM cells from asthmatic and non-asthmatic donors. This
811 is characterised by increased RNA polymerase II, C/EBP β and p65 binding (previously
812 published); aberrant presence of H3K18Ac and p300 at the asthmatic promoter. CpG methylation
813 decreases as proximity to the transcription start site increases but is not different between ASM
814 cells from asthmatic and non-asthmatic donors. CXCL8 transcription is dependent on the
815 presence of histone acetylation reader proteins Brd3 and Brd4 and BET protein inhibitors can
816 reduce asthmatic CXCL8 expression to non-asthmatic levels via disruption of Brd4 and RNA
817 polymerase II association with the promoter.

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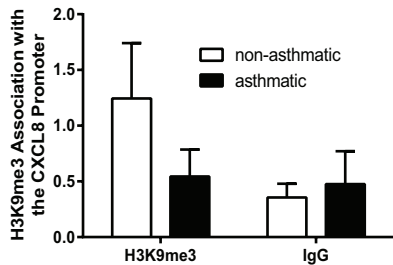
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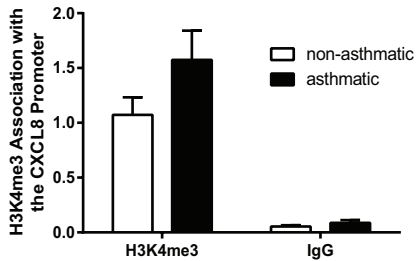
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Figure 1

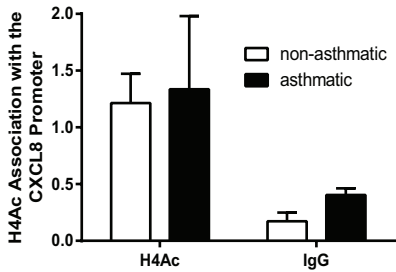
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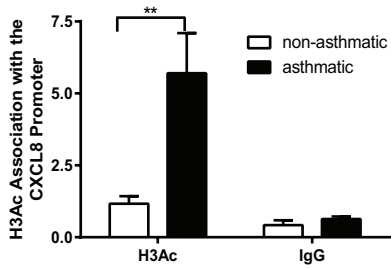
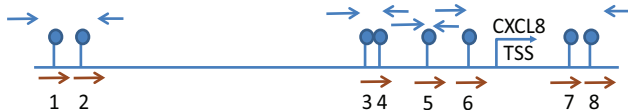


Figure 2

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B

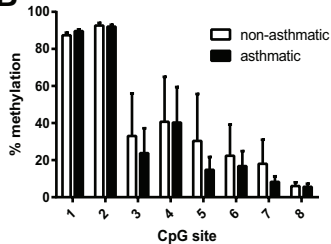


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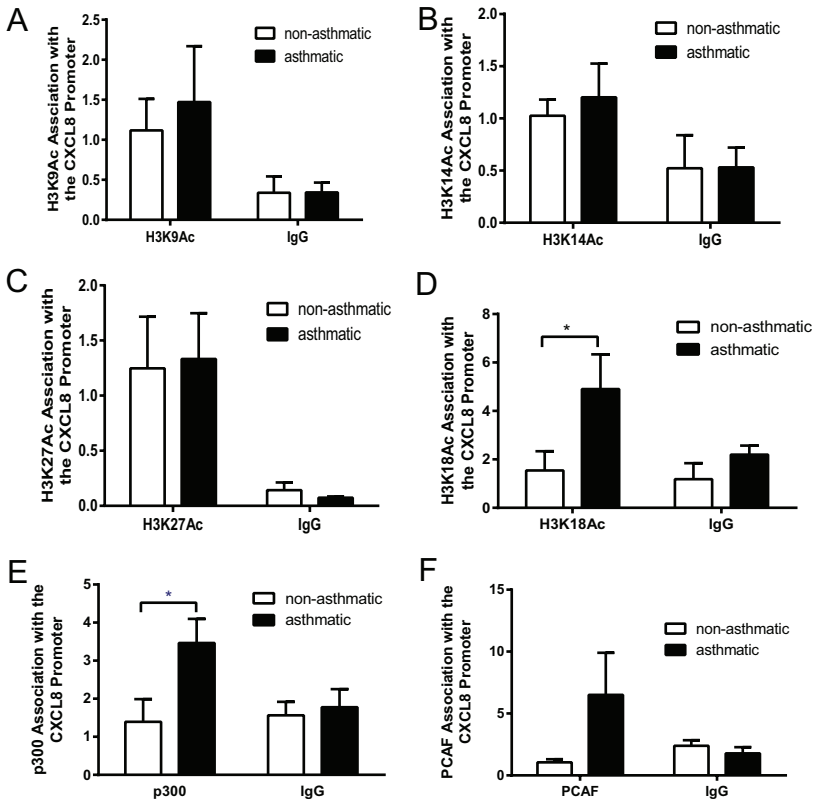


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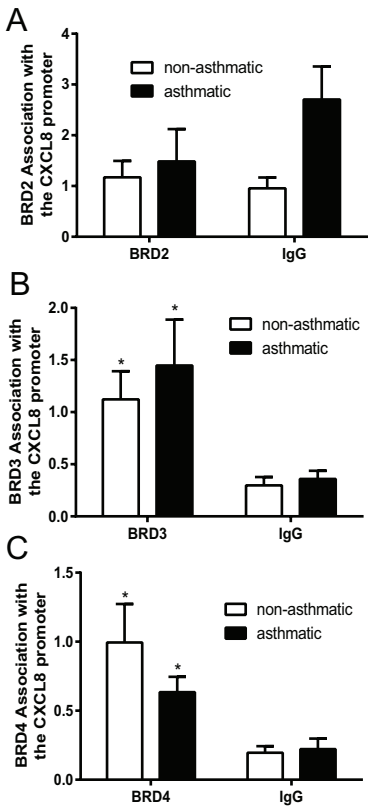


Figure 5

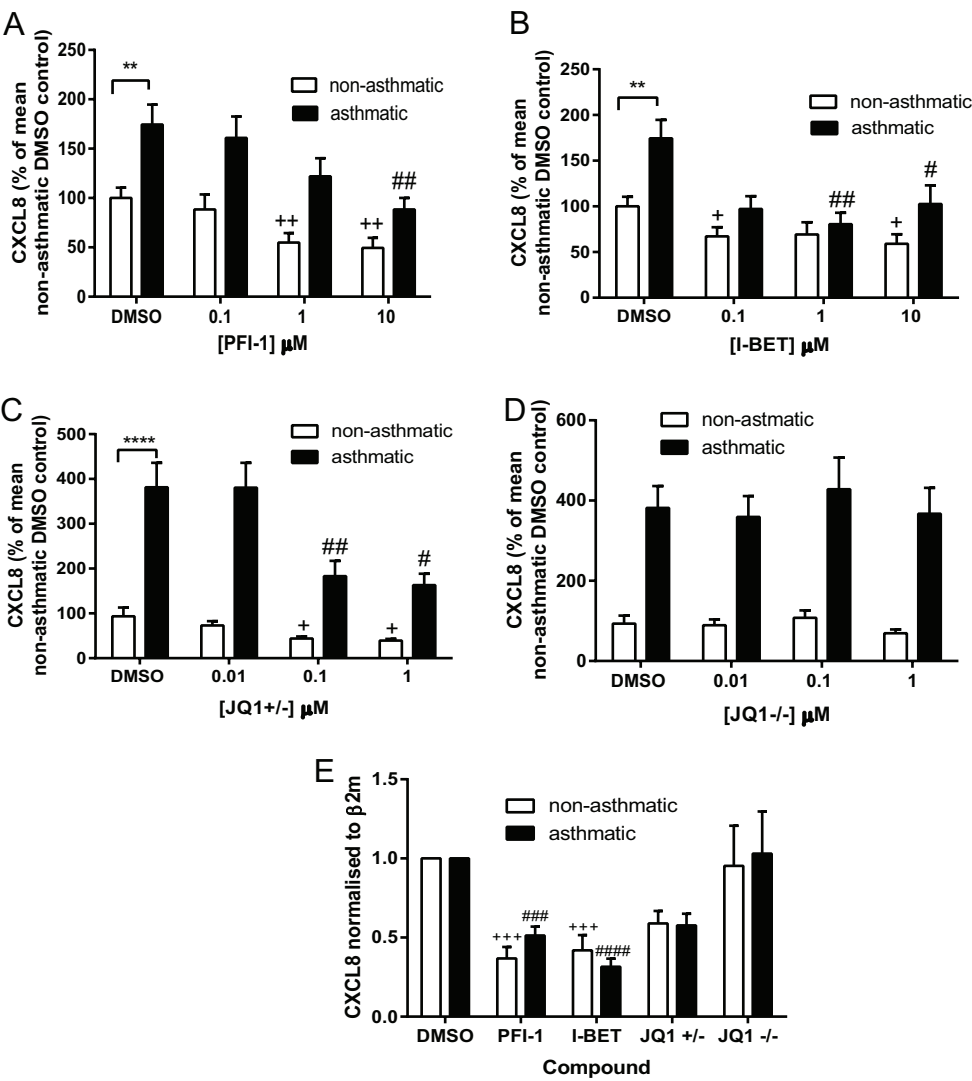
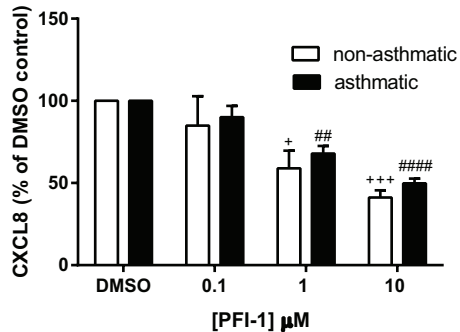
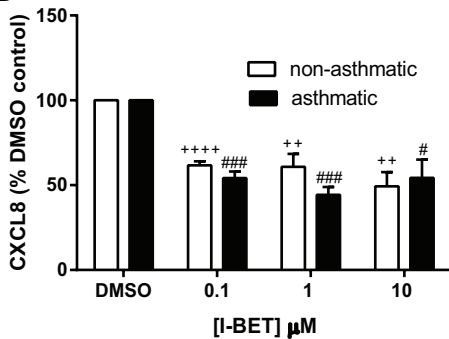


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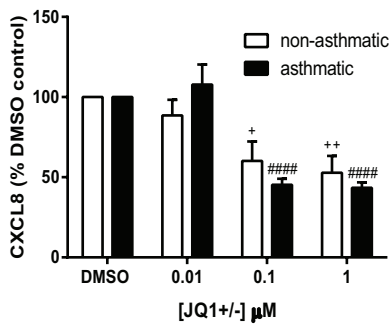
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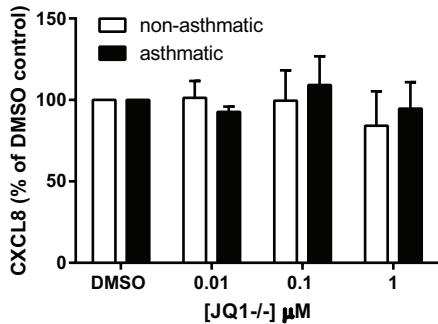
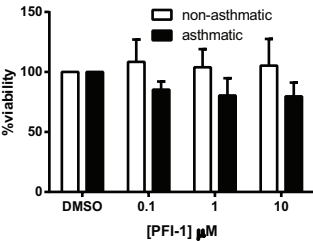
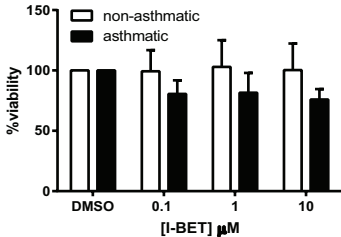


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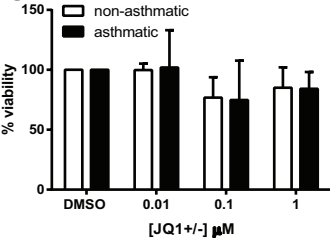
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D

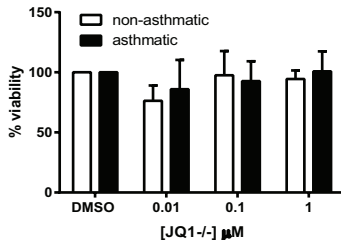
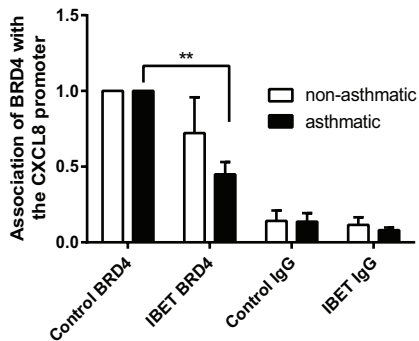
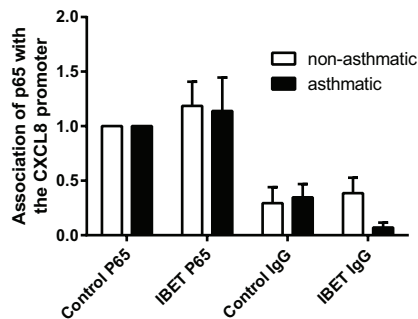


Figure 8

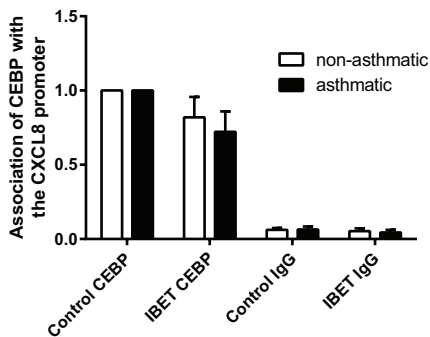
A



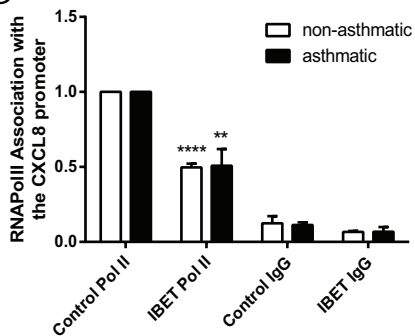
B



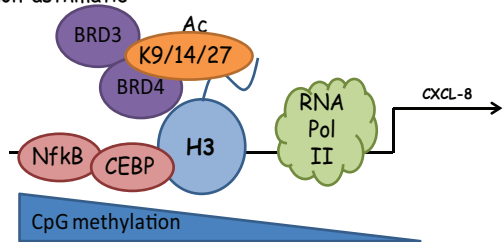
C



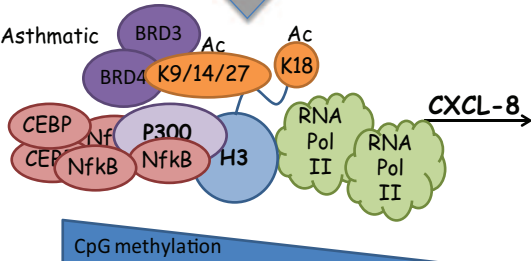
D



Non-asthmatic



Asthmatic



+ BET inhibitor

Asthmatic

