Am J Physiol Lung Cell Mol Physiol 311: L421–L432, 2016.
First published July 1, 2016; doi:10.1152/ajplung.00179.2016.

Glucocorticoid and TNF signaling converge at A20 (TNFAIP3) to repress airway smooth muscle cytokine expression

Sarah K. Sasse,[1] Mohammed O. Altonsy,[2] Vineela Kadiyala,[1] Gaoyuan Cao,[3] Reynold A. Panettieri Jr,[3] and Anthony N. Gerber[1,4]

[1]*Department of Medicine, National Jewish Health, Denver, Colorado;* [2]*Department of Zoology, Sohag University, Sohag, Egypt;* [3]*Rutgers Institute for Translational Medicine & Science, Rutgers University, New Brunswick, New Jersey; and* [4]*Department of Medicine, University of Colorado, Denver, Colorado*

Submitted 4 May 2016; accepted in final form 29 June 2016

Sasse SK, Altonsy MO, Kadiyala V, Cao G, Panettieri RA Jr, Gerber AN. Glucocorticoid and TNF signaling converge at A20 (TNFAIP3) to repress airway smooth muscle cytokine expression. *Am J Physiol Lung Cell Mol Physiol* 311: L421–L432, 2016. First published July 1, 2016; doi:10.1152/ajplung.00179.2016.—Airway smooth muscle is a major target tissue for glucocorticoid (GC)-based asthma therapies, however, molecular mechanisms through which the GC receptor (GR) exerts therapeutic effects in this key airway cell type have not been fully elucidated. We previously identified the nuclear factor-κB (NF-κB) inhibitor, A20 (TNFAIP3), as a mediator of cytokine repression by glucocorticoids (GCs) in airway epithelial cells and defined cooperative regulation of anti-inflammatory genes by GR and NF-κB as a key mechanistic underpinning of airway epithelial GR function. Here, we expand on these findings to determine whether a similar mechanism is operational in human airway smooth muscle (HASM). Using HASM cells derived from normal and fatal asthma samples as an in vitro model, we demonstrate that GCs spare or augment TNF-mediated induction of A20 (*TNFAIP3*), *TNIP1*, and *NFKBIA*, all implicated in negative feedback control of NF-κB-driven inflammatory processes. We applied chromatin immunoprecipitation and reporter analysis to show that GR and NF-κB directly regulate A20 expression in HASM through cooperative induction of an intronic enhancer. Using overexpression, we show for the first time that A20 and its interacting partner, TNIP1, repress TNF signaling in HASM cells. Moreover, we applied small interfering RNA-based gene knockdown to demonstrate that A20 is required for maximal cytokine repression by GCs in HASM. Taken together, our data suggest that inductive regulation of A20 by GR and NF-κB contributes to cytokine repression in HASM.

airway smooth muscle; cytokine; glucocorticoid; NF-κB; tumor necrosis factor

DYSFUNCTION IN AIRWAY SMOOTH MUSCLE (ASM) is central to asthma pathophysiology (18, 44, 47). Alterations in ASM contractile function have long been recognized as a major contributor to episodic shortness of breath in asthma (35). More recently, it has been recognized that ASM is also a rich source of cytokines that signal to other cell types within the airway (13), and thus contributes to asthma symptoms through promoting inflammation (23). Glucocorticoids (GCs), which are the centerpiece of current asthma treatment guidelines (46), are believed to reduce airway hyperresponsiveness and repress ASM remodeling (16, 17, 58). In addition to these effects on ASM physiology and structure, as is the case in many cell types, GCs potently repress ASM cytokine expression (22). Consequently, ASM is now viewed as a key target of GC-based anti-inflammatory therapy in asthma and as a potential nidus for steroid-resistant inflammation.

Across tissue types and diseases, including asthma, there is controversy regarding the relative contributions of direct cytokine repression vs. activation of anti-inflammatory genes by GCs in mediating therapeutic effects (10). The glucocorticoid receptor (GR) is a nuclear receptor that, upon ligand binding, is induced to translocate to the nucleus and regulate gene expression. GR can form homodimers that exhibit energetically favorable interactions with palindromic or semipalindromic GR-binding sequences, which have been linked to GC-mediated gene induction (41). However, typical binding sites for dimeric GR are not always present within regulatory regions for cytokines subject to GC-mediated repression (53), and a mutation that disrupts GR dimerization did not appear to substantially affect repression of a canonical nuclear factor-κB (NF-κB)-driven reporter (6). These and other data have led to the notion that GR dimerization and association of dimeric GR with high-affinity GR-binding sites is not required for cytokine repression by GCs. Instead, the dominant model for GR-mediated repression has centered on repressive tethering interactions between monomeric GR and inflammatory transcription factors, leading to gene inactivation in association with histone deactylation (4, 15, 57). More recently, however, it has become clear that mutations in the GR dimerization domain do not completely abrogate gene induction by GR, nor do mice harboring the GR dimerization mutation exhibit normal anti-inflammatory responses (19, 32, 54, 61). Moreover, a number of GR-induced targets, such as DUSP1, have emerged as being crucial for GR-mediated inflammatory repression in many tissues, including ASM (31, 49). Thus the precise mechanisms through which GR represses inflammation remain to be fully elucidated in many tissues and diseases, including asthma.

Major inflammatory signaling pathways relevant to asthma such as IL-1A, TNF, TLR4, and IL-13, induce potent negative feedback responses that are crucial for normal termination of inflammation (37, 64, 67). In addressing the mechanistic basis for GR-mediated cytokine repression in airway epithelial cells, we previously found that GR differentially regulates pro- and anti-inflammatory targets of NF-κB, a major common downstream transcriptional effector of inflammatory cascades driven by diverse stimuli. This differential regulation was due to cooperation between GR and NF-κB at enhancers for genes implicated in repressing inflammation, best exemplified by combined treatment of Beas-2B cells with TNF and dexamethasone (dex), a potent GR agonist, resulting in synergistic induction of an intronic enhancer for A20/TNFAIP3 (1). A20

Address for reprint requests and other correspondence: A. N. Gerber, Dept. of Medicine, National Jewish Health, Rm. K621, 1400 Jackson St, Denver, CO 80206 (e-mail: gerbera@njhealth.org).

is a powerful negative feedback inhibitor of NF-κB that is induced by a host of inflammatory pathways and acts through a nondegradative ubiquitin-based mechanism to curtail NF-κB activity (12, 38, 65). A20 dysfunction has been genetically linked to a variety of immune-mediated diseases, including asthma, and A20 has also been shown to limit inflammation in models of allergic airway inflammation and tolerance (30, 56). Moreover, we have recently shown that A20 is required for full cytokine repression by GCs in airway epithelial cells (28), implicating GR cooperation with NF-κB as an important contributor to steroid efficacy. However, expression and function of A20 and its interacting partner, TNIP1 (62), have not been investigated in human airway smooth muscle (HASM). Whether GR cooperates with NF-κB in ASM also remains to be elucidated.

In this study, we used cultured ASM derived from normal controls and from patients with fatal asthma (FA) to determine whether GC signaling selectively spares the expression of putative anti-inflammatory genes in ASM. We applied chromatin immunoprecipitation (ChIP) and reporter assays to determine whether GR and NF-κB coinduce anti-inflammatory gene expression. We used adenoviral overexpression and small interfering RNA (siRNA) knockdown to test whether A20 and TNIP1 repress cytokine expression in HASM cells. Our data implicate cooperative induction of A20 by GR and p65 as a novel transcriptional mechanism contributing to steroid-mediated cytokine repression in HASM.

MATERIALS AND METHODS

Cell culture and reagents. Primary HASM cells were derived from two donors with normal ASM (HASM1, a 20-yr-old man; HASM2, a 54-yr-old woman) and two patients with FA (FA-HASM1, a 15-yr-old girl; FA-HASM2, a 48-yr-old woman) as described previously (3). No recent corticosteroid use was noted for any of the donors. Phenotypic characteristics of ASM were confirmed by microscopy and were similar between the lines, although FA-HASM2 cells were somewhat larger than the other three lines. Cells were cultured in Ham's F-12 medium with L-glutamine (Corning) supplemented with 10% FBS (Fisherbrand) and 1% penicillin/streptomycin (Corning), and maintained in 5% CO_2 at 37°C. Recombinant human TNFα (PHC3015L; Life Technologies) was diluted in sterile 1× Dulbecco's PBS containing 0.2% FBS and used at a final concentration of 20 ng/ml. Dexamethasone (dex; D1756; Sigma) was dissolved in sterile ethanol and used at a final concentration of 100 nM. Salbutamol sulfate (salb, S2507; Selleck Chemicals) was dissolved in sterile cell-culture grade water and used at a final concentration of 1 μM. Adenoviral expression constructs for TNFAIP3 (Ad-*TNFAIP3*) and green fluorescent protein (Ad-*GFP*) have been described previously (1). The TNIP1 adenoviral expression construct (Ad-*TNIP1*) was obtained from Welgen and uses TNIP1 cDNA BC014008. siRNA-mediated knockdown was performed using ON-TARGETplus SMARTpool siRNA against TNFAIP3 (si-*TNFAIP3*, L-009919-00-0005) and a nontargeting control (si-*Ctrl*; D-001810-10-20) supplied by GE Healthcare/Dharmacon. Primary antibodies for Western blotting included anti-TNFAIP3 (ab13597), anti-TNIP1 (ab90890), anti-IL-1β (ab156791), and anti-beta-actin (ab75186) from Abcam, and anti-GAPDH (sc-25778) from Santa Cruz Biotechnology. Secondary antibodies were enhanced chemiluminescence (ECL) sheep anti-mouse IgG-horseradish peroxidase (HRP) (95017-332) and ECL donkey anti-rabbit IgG-HRP (95017-330) supplied by VWR. Antibodies used in ChIP experiments included anti-GR (IA-1, a generous gift from Dr. Miles Pufall) and anti-NF-κB p65 (sc-372x) from Santa Cruz Biotechnology.

RNA purification and quantitative PCR analysis. For gene expression analysis, HASM and FA-HASM cells were plated in 6-well plates at a density of approximately $4–5 \times 10^5$ cells/well. Once confluent, cells were treated with vehicle, TNF, dex, or TNF + dex for 4 h, after which cells were lysed in TRIzol reagent (Life Technologies), and RNA was purified using the PureLink RNA Mini Kit (Life Technologies). Reverse transcription and quantitative PCR (qPCR) were performed as previously described (55). Relative gene expression was calculated by normalizing the mean C_T value for each gene to the mean C_T value for RPL19 and is expressed on a $\log_2$ scale relative to the mean normalized C_T value of vehicle-treated samples. Primer sequences used for qPCR are listed in Table 1.

Western blotting and ELISA. To assess protein expression by Western blot analysis following drug treatment, adenoviral transduction, and/or siRNA-mediated knockdown, HASM2 or FA-HASM1 cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors, and then 20–50 μg of total protein were separated by SDS-PAGE and transferred onto Immun-Blot polyvinylidine difluoride membranes from Bio-Rad. Membranes were probed with antibodies targeting proteins of interest, and bands were visualized using the ECL Prime detection system from GE Healthcare. To measure secreted CXCL8 protein levels, supernatants from HASM2 and FA-HASM1 cells treated for 24 h with vehicle, TNF, dex, or TNF + dex were assayed using a Human CXCL8/IL-8 DuoSet ELISA and Ancillary Reagent Kit 2 (both from R&D Systems) according to the manufacturer's protocols.

ChIP-qPCR. For each treatment group of a ChIP experiment, HASM2 cells were plated at a density of $\sim 1 \times 10^6$ cells/plate on three 15-cm plates and grown to confluence. Cells were treated for 1 h with vehicle, TNF, dex, or TNF + dex, and then fixed in 1% formaldehyde for 5 min. Chromatin was isolated as previously described (55), divided into five biological replicate samples per treatment group, and then sonicated on high power for 25 cycles (1 cycle = 30 s on, 30 s off) in a Bioruptor from Diagenode. Immunoprecipitation using antibodies against GR or p65 was performed as described (55) and qPCR was used to assay relative factor occupancy at genomic target regions, which was calculated as the difference between the mean C_T value for the target region and the geometric mean of the C_T values at three nonoccupied negative control regions, and is expressed on a $\log_2$ scale. Primer sequences used for ChIP-qPCR are listed in Table 1.

Plasmid transfection and reporter assays. The p*TNFAIP3*, p*TNFAIP3* mut GR binding sequence (GBS), and p*FKBP5* luciferase reporter plasmids were constructed in the pGL3-Promoter backbone (Promega) and have been described previously (1). pSV40-mCherry has also been previously described (20). For reporter assays, HASM2 or FA-HASM1 cells were plated in 48-well plates at a density of 1×10^5 cells/well. Three days later, cells were transfected with 250 ng of total plasmid DNA, consisting of a 10:1:1 ratio of firefly luciferase reporter construct, *Renilla* luciferase control construct (pSV40-RL from Promega), and pSV40-mCherry for visualization of transfection efficiency using Lipofectamine 3000 and accompanying P3000 Reagent (Life Technologies) according to the manufacturer's protocol. Approximately 18 h later, cells were treated as indicated for 8 h, and reporter activity was assayed as detailed previously (55).

Adenoviral transduction. For adenoviral transduction experiments, HASM2 cells were plated in 6-well plates at a density of approximately $4–5 \times 10^5$ cells/well and grown to confluence. For TNFAIP3 overexpression experiments, cells were transduced with Ad-*TNFAIP3* or Ad-*GFP* at a multiplicity of infection (MOI) of 100. For TNIP1 transduction experiments, Ad-*TNIP1* and Ad-*GFP* were used at an MOI of 50. Approximately 17 h following adenoviral infection, cells were treated with vehicle, TNF, dex, or TNF + dex for 4 h, and gene expression was assayed via qPCR.

siRNA transfection. HASM2 cells were plated in antibiotic-free medium for siRNA-mediated knockdown experiments in 6-well plates at a density of 5×10^5 cells/well for qPCR analysis or in 6-cm plates at 9×10^5 cells/plate for protein expression analysis. The next day,

Table 1. *Sequences of primers used for qPCR and ChIP-qPCR*

	Forward Sequence (5′ to 3′)	Reverse Sequence (5′ to 3′)
qPCR primers		
IL1A	CATCCTCCACAATAGCAGACAG	GAGTTTCCTGGCTATGGGATAAG
IL1B	CAAAGGCGGCCAGGATATAA	CTAGGGATTGAGTCCACATTCAG
IL6	TGACCCAACCACAAATGC	AGGAACTCCTTAAAGCTGCG
CXCL8	CTTGGCAGCCTTCCTGATTT	GGGTGGAAAGGTTTGGAGTATG
HBEGF	TCTGGACCTTTTGAGAGTCACTTTATC	CGTGCTCCTCCTTGTTTGGT
TNF	AGAGGGAGAGAAGCAACTACA	GGGTCAGTATGTGAGAGGAAGA
TNFAIP3 5'-UTR	AGTGTTCCCAGGTGGCCTTAGAAA	TCTCAGCCAAGACGATGAAGCAGT
TNFAIP3 exon 5	GCCCAGGAATGCTACAGATAC	AGTGGAACAGCTCGGATTTC
TNIP1	AACAAGCAGTGGGACCAGCATTTC	TGCTTCTGCAAATCAGCCAGCTTC
TNIP2	TCTGCCTCGAACCAGTCAAATGGA	AACTTCCGGCTGGATGACAAATGC
NFKBIA	GAGTTACCTACCAGGGCTATTC	CTCTCCTCATCCTCACTCTCT
ChIP-qPCR primers		
FKBP5	TAACCACATCAAGCGAGCTG	GCATGGTTTAGGGGTTCTTG
IL1A	GTGTATGCCTGTGTCACATAAAC	GCATTTGCAACCACACCTAAT
IL1B	GATGGAGGCTCAGGTCTAATG	CGTGTCTCAGGCTCTCATTT
IL6	CGTGCATGACTTCAGCTTTAC	AGCAGAACCACTCTTCCTTTAC
CXCL8	GAGCACTCCATAAGGCACAA	TTCCTTCCGGTGGTTTCTTC
TNFAIP3	GACCACACCCACTTGGAAA	TTTGACTAGCAATTGAGCAACAG
NFKBIA	CCTTCCTCAACTTCCAGAACA	GTGACTCTGCTACATCAGCTAC
TNIP1 pair1	GACCGAGCGAGGTAAACAC	CAGGGAAGTCGCTCCTAGA
TNIP1 pair2	ACTAATACAGGGTGGCATGTT	CCTTTGCTTCCACTTCCTTTG
Negative controls		
MYOD1	TGCAGGAGATGAAATACTAAGCAAGTA	AGATTGGAAACTGAGGACTTTAGTTAGAG
GAPDH	CGGCTACTAGCGGTTTTACG	AAGAAGATGCGGCTGACTGT
OLIG3	GGCAAGGACAGAGACAATCATA	CTCTGTGTTCTCGCTTTGGA

ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.

cells were transfected with 20 nM si-*TNFAIP3* or si-*Ctrl* using a modified protocol for Lipofectamine 2000. Briefly, siRNA and Lipofectamine transfection reagent were first diluted and subsequently complexed in antibiotic- and serum-free medium. Wells/plates were then rinsed with supplement-free medium and cells were incubated with the diluted complex for 6 h. Additional serum-containing medium was then added to the transfection medium to adjust the concentration of FBS to 10%, and cells were incubated overnight. Medium was replaced with fresh complete medium the following morning, and cells were given a day to recover before being treated with vehicle, TNF, dex, or TNF + dex for an additional 24 h. Gene and protein expression were analyzed by qPCR and Western blotting, respectively.

Statistical analysis. A Student's *t*-test was applied to make statistical comparisons as indicated in figure legends. Note that comparisons for different treatment conditions are based on biologic replicates within each cell line (generally four), as opposed to comparing the data between the cell lines.

RESULTS

Glucocorticoids differentially regulate pro- and anti-inflammatory genes induced by TNF in ASM. To examine GC regulation of TNF-induced pro- and anti-inflammatory genes in ASM, cultured primary HASM cells from two subjects with normal ASM (HASM1 and HASM2) were treated with TNF, dex (a potent synthetic GR ligand), or TNF + dex for 4 h. As illustrated in Figure 1, *A* and *B* (*top*), TNF induction of classic proinflammatory targets such as *IL1A*, *IL1B*, and *IL6* was strongly repressed by cotreatment with dex in both HASM lines. In contrast, induction of TNF targets such as *TNFAIP3*, *TNIP1*, and *NFKBIA*, which have documented anti-inflammatory function in other cell types (51), was maintained or augmented by the addition of dex (Fig. 1, *A* and *B*, *bottom*). The effects of dex on pro- vs. anti-inflammatory gene expression were statistically different in both lines on the basis of rank-sum test comparisons of expression changes within the two gene groups ($P < 0.05$). Similarly statistically distinguishable outcomes of dex cotreatment on pro- vs. anti-inflammatory gene induction by TNF were observed in HASM cells derived from two patients with FA (FA-HASM1 and FA-HASM2) and treated as described above (Fig. 1, *C* and *D*, respectively). Thus consistent with our previous findings in BEAS-2B and normal primary human airway epithelial cells (1, 28), induction of proinflammatory TNF target gene expression is suppressed by GCs, whereas induction of anti-inflammatory targets is selectively spared or augmented in ASM.

The effects of dex on TNF-mediated gene regulation were further assayed by Western blot analysis of TNFAIP3 and TNIP1 both in normal (HASM2) and FA (FA-HASM1) cells. These protein data indicate that dex also spares the expression of anti-inflammatory TNF targets at the protein level (e.g., TNFAIP3 in HASM2 and TNIP1 in FA-HASM1; Fig. 1, *E* and *F*, respectively). Secreted levels of CXCL8 were also measured using ELISA. This showed that TNF-induced CXCL8 secretion was reduced by dex in HASM2, but not in FA-HASM1. These data are consistent with prior reports of abnormal CXCL8 regulation in ASM from patients with asthma (27).

GR and NF-κB co-occupancy of genomic binding sites mediates GC regulation of both pro- and anti-inflammatory TNF targets in ASM. GR occupancy is strongly dependent on cellular context (21, 50), and it is possible that selective repression of proinflammatory targets of NF-κB in ASM is due to differential GR occupancy at regulatory elements mediating pro- and anti-inflammatory gene expression. Therefore, to explore the mechanistic basis for distinct outcomes of GR–NF-κB cross-talk at pro- vs. anti-inflammatory TNF target genes in HASM cells, we used ChIP-qPCR to analyze the

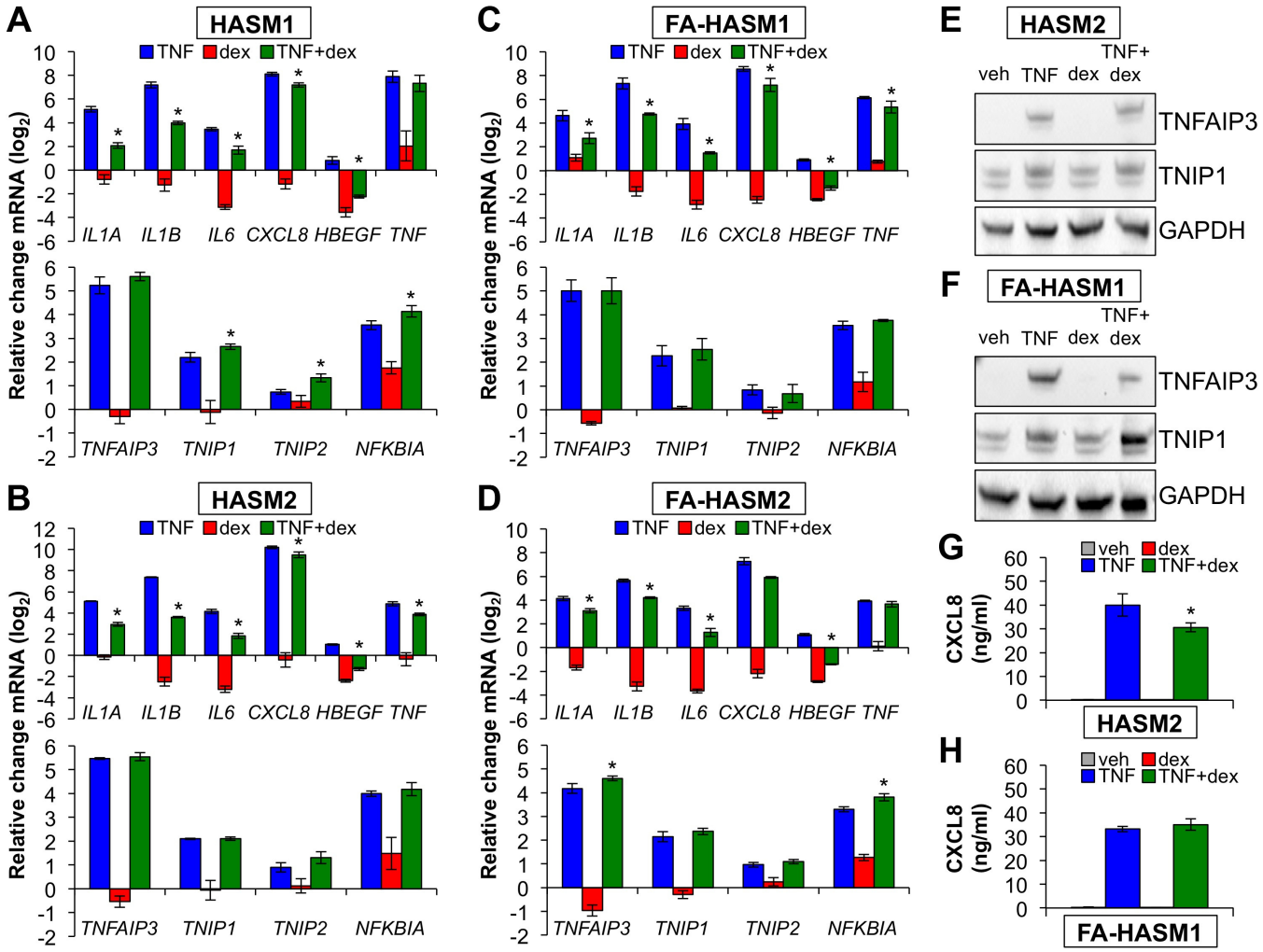

Fig. 1. Glucocorticoids differentially regulate pro- vs. anti-inflammatory gene induction by tumor necrosis factor (TNF) in airway smooth muscle (ASM). *A–D*: primary human ASM cells derived from two subjects with normal ASM (HASM; *A* and *B*) and two patients with fatal asthma (FA-HASM; *C* and *D*) were treated with TNF, dexamethasone (dex), or TNF + dex for 4 h and expression of proinflammatory (*top*) and anti-inflammatory (*bottom*) genes was assayed using quantitative PCR (qPCR). Bars indicate mean normalized C_T values on a $\log_2$ scale (+SD) relative to vehicle-treated controls. *$P \leq 0.05$ compared with response of same gene in TNF-treated cells. *E* and *F*: Western blots of TNFAIP3 and TNIP1 protein expression in HASM (*E*) and FA-HASM (*F*) cells treated as indicated for 24 h. GAPDH was used as a loading control. *G* and *H*: secreted CXCL8 levels in the supernatants of HASM (*G*) and FA-HASM1 (*H*) cells treated as indicated for 24 h, as measured by ELISA. *$P \leq 0.05$ compared with response in TNF-treated cells.

occupancy of GR and the p65 subunit of NF-κB at genomic binding sites previously identified in other cell types (1, 28, 50). Schematics of the regions interrogated for occupancy of both factors are depicted in Figure 2*A*. Antibodies against GR or p65 were used to immunoprecipitate factor-bound chromatin fragments prepared from HASM2 cells treated for 1 h with vehicle, TNF, dex, or TNF + dex, as indicated in Figure 2, *B–D*. Factor occupancy, expressed on a $\log_2$ scale, was calculated relative to the geometric mean of a panel of nonoccupied negative control regions. We first determined GR and p65 occupancy at two control regions (Fig. 2*B*, *top* and *bottom*, respectively), including *MYOD1*, a negative control region that is not occupied by either factor with TNF or dex stimulation, and *FKBP5*, a well-characterized site of GR recruitment following dex exposure. Indicative of high ChIP efficiency, neither GR nor p65 exhibited measurable occupancy at *MYOD1* under any treatment conditions, whereas GR occupancy was markedly increased at *FKBP5* by dex treatment. We next interrogated GR and p65 occupancy within a set of proinflammatory TNF targets, represented by classic cytokines such as *IL1B* and *CXCL8* (Fig. 2*C*), and a set of anti-inflammatory TNF targets, including *TNFAIP3* and *TNIP1* (Fig. 2*D*). Consistent with moderate mRNA levels for many TNF targets, including cytokines such as IL-6, under basal culture conditions (39), p65 occupancy was detected with vehicle treatment at most tested sites. TNF treatment, however, led to significant increases in p65 occupancy at most sites, which was generally maintained with TNF + dex cotreatment (Fig. 2, *C* and *D*, *bottom*). The effects of dex treatment alone on GR occupancy were more varied (Fig. 2, *C* and *D*, *top*). In some cases there was detectable GR occupancy with vehicle or TNF treatment alone, consistent with other recent reports of nuclear localization of so-called unliganded GR in ASM (8, 26). Regardless of the magnitude of inductive effects of TNF or dex alone on GR occupancy, however, TNF + dex cotreatment resulted in generally similar patterns and levels of GR binding at interro-

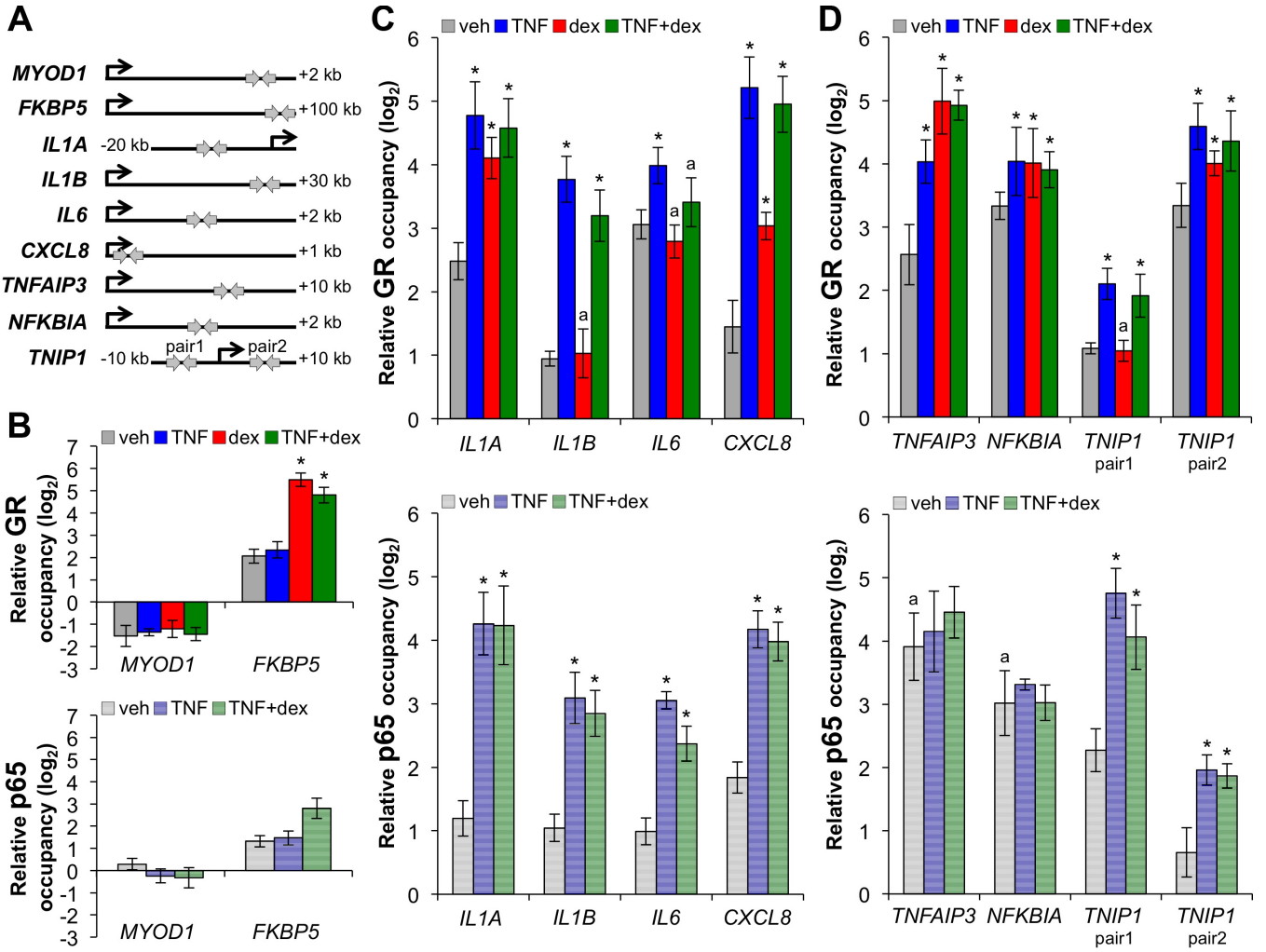

Fig. 2. Glucocorticoid regulation of both pro- and anti-inflammatory TNF target genes in ASM is associated with glucocorticoid receptor (GR) and NF-κB occupancy of genomic binding sites. *A*: schematic diagram illustrating approximate genomic positions targeted by chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) primers referred to in *B–D*. Large black arrows represent the transcription start site, defined as the first nucleotide of each gene's 5′-untranslated region or first exon, and light gray opposing arrowheads show the approximate location of the primer pairs. Each locus is drawn to scale, with adjacent numbers indicating total distance [in kilobases (kb)] relative to each TSS. Primer pairs span a region of approximately 100–200 bp of genomic DNA. *B–D*: ChIP-qPCR analysis of GR (*top*, solid bars) and p65 (*bottom*, shaded bars) occupancy in HASM2 cells following a 1-h incubation with vehicle (veh), TNF, dex, or TNF + dex, as indicated. Bars represent factor occupancy on a $\log_2$ scale (+SD), expressed as the mean C_T value at each target region relative to the geometric mean of C_T values at three negative control regions. Relative GR and p65 occupancy is shown at negative (*MYOD1*) and positive (*FKBP5*, a canonical GR target) control sites (*B*) and both pro- (*C*) and anti-inflammatory (*D*) loci. *$P \leq 0.05$ vs. vehicle, [a]$P \leq 0.05$ vs. negative controls.

gated regions within the pro- and anti-inflammatory gene sets. Thus differential regulation of pro- and anti-inflammatory targets in HASM cells does not appear to be a consequence of differential factor occupancy.

GR–NF-κB cooperative induction of TNFAIP3 enhancer activity is facilitated by the β-adrenoceptor agonist salbutamol and requires an intact GR binding site. Although GR is widely understood to repress NF-κB activity, we and others have collectively identified regulatory elements in which GR and p65 appear to cooperatively enhance transcription in several cell types (28, 43, 63). To test whether a similar mechanism operates in HASM, we transfected HASM2 and FA-HASM1 cells with a *TNFAIP3* luciferase reporter (p*TNFAIP3*) that we have shown exhibits cooperative regulation by GR and NF-κB in airway epithelial cells (1). Cells were subsequently treated with vehicle, TNF, dex, or TNF + dex for 8 h and harvested for luciferase assays. Because β-adrenoceptor agonists are frequently combined with GCs in clinical treatment of airway diseases (34), we also explored the effects of the β-adrenoceptor agonist, salbutamol, on p*TNFAIP3* reporter activity. As shown in Figure 3, *A* and *B*, p*TNFAIP3* showed modest induction with TNF and dex treatments alone that was markedly augmented by TNF + dex cotreatment. Moreover, addition of salbutamol to TNF + dex cotreatment resulted in further enhancement of p*TNFAIP3* activity beyond that induced by TNF + dex. In contrast, a reporter containing the *FKBP5* regulatory region targeted by our ChIP-qPCR primers (p*FKBP5*) exhibited dex-inducible activity that was repressed by TNF; augmentation of dex-mediated induction by salbutamol was also repressed by TNF. Thus TNF can augment or repress the effects of GCs in a reporter-specific manner.

Gene induction by dimeric GR is generally implicated in mediating steroid side effects as opposed to contributing to

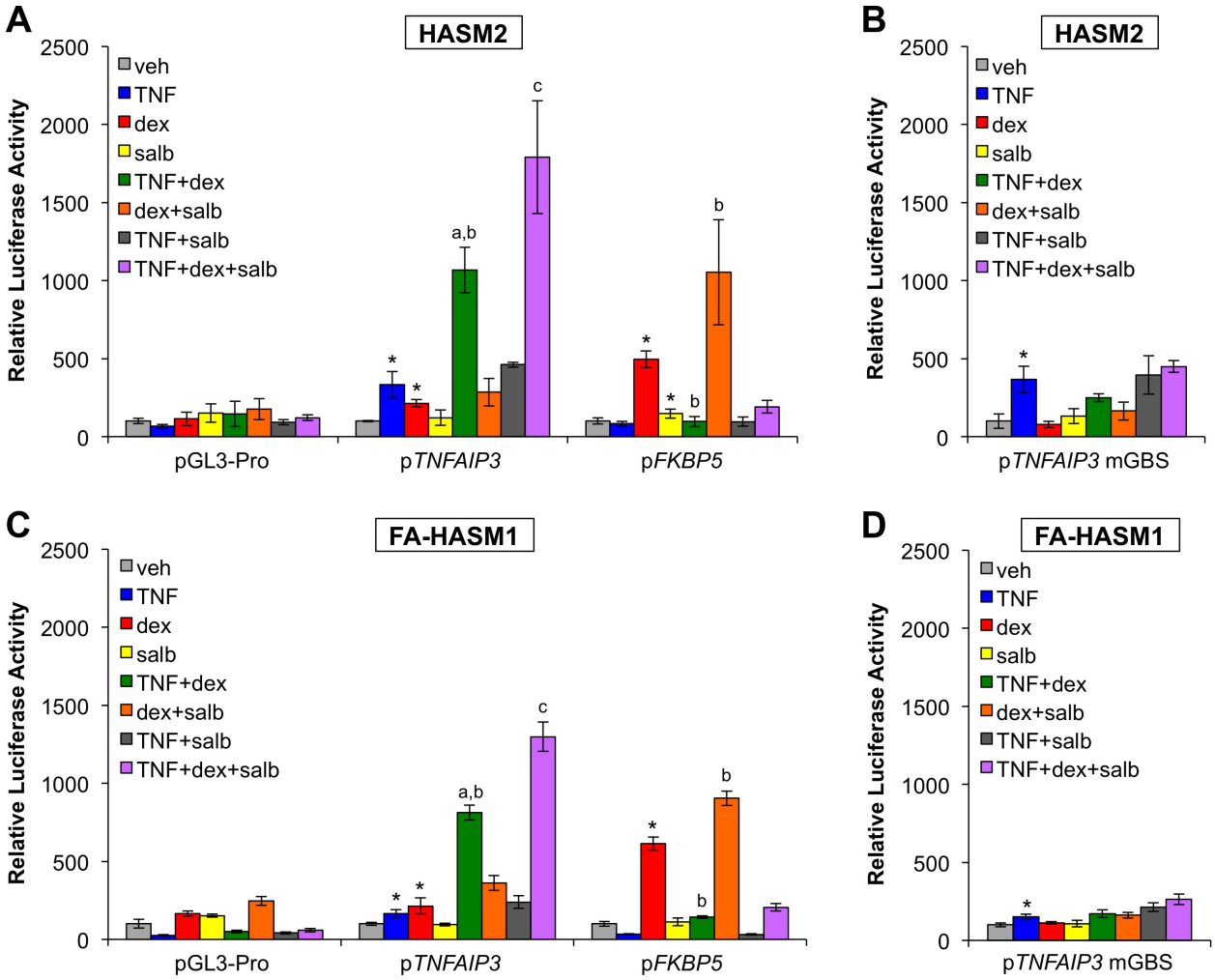

Fig. 3. Synergistic induction of TNFAIP3 enhancer activity by GR and nuclear factor-κB (NF-κB) in ASM is further augmented by salbutamol (salb) and requires an intact GR binding site. *A–D*: luciferase activity of reporter constructs or pGL3-Promoter empty vector control (pGL3-Pro) transiently transfected into HASM2 (*A* and *B*) or FA-HASM1 (*C* and *D*) cells prior to treatment with veh, TNF, dex, salb, or various combinations thereof, as indicated, for 8 h. Activation of each reporter was normalized to that of a control *Renilla* luciferase construct and is expressed relative to its activity in vehicle-treated cells. Bars represent means (+SD). $^{*}P \leq 0.05$ vs. vehicle, $^{a}P \leq 0.05$ vs. TNF, $^{b}P \leq 0.05$ vs. dex, $^{c}P \leq 0.05$ vs. TNF + dex.

anti-inflammatory gene regulation (14). Therefore, we examined the role of a conserved semipalindromic GBS, which is predicted to interact strongly with dimeric GR, in mediating combinatorial induction of p*TNFAIP3* by TNF, dex, and/or salbutamol in HASM. We accomplished this by testing the inducibility of a mutant construct, p*TNFAIP3* mGBS, in which this GBS had been disrupted as previously described (1). Although the GBS mutant reporter maintained responsiveness to TNF, all additional inductive effects of dex and/or salbutamol cotreatment were completely lost (Fig. 3, *C* and *D*). These results implicate GR interaction with this dimeric GBS as being central to the molecular mechanism underlying cooperative induction of *TNFAIP3* by TNF + dex and its enhancement by salbutamol.

Dose-response effects of dex on expression of TNFAIP3, FKBP5, and IL-6. The data described above indicate that GR and p65 cooperatively induce an intronic enhancer of A20, but do not determine a relationship between the dose of dex and cooperation between GR and p65. We therefore performed dose-response experiments examining both the regulation of the endogenous *TNFAIP3* gene by qPCR and the *TNFAIP3* reporter. HASM2 (Fig. 4*A*) and FA-HASM1 (Fig. 4*B*) cells were exposed to dex doses ranging between 1 and 100 nM for 4 h with and without TNF cotreatment. Expression levels of *FKBP5*, *IL6*, and *TNFAIP3* were determined by qPCR. Whereas regulation of both *IL6* and *FKBP5* exhibited a clear dose response to dex, and repression of *TNFAIP3* by dex without TNF treatment was also dose dependent, all tested doses of dex spared *TNFAIP3* expression in both cell lines. These data are consistent with our data in airway epithelial cells in which the effect of dex on *TNFAIP3* expression appears to integrate both cooperation with p65 at the *TNFAIP3* intronic enhancer and antagonism of p65 activity at other enhancers within the *TNFAIP3* locus. Supporting this notion, the p*TNFAIP3* luciferase reporter showed a dose-dependent increase in activity (Fig. 4, *C* and *D*), which generally mirrored the dose-dependent response of p*FKBP5* to dex, although p*TNFAIP3* appeared to be somewhat more sensitive to lower dex doses.

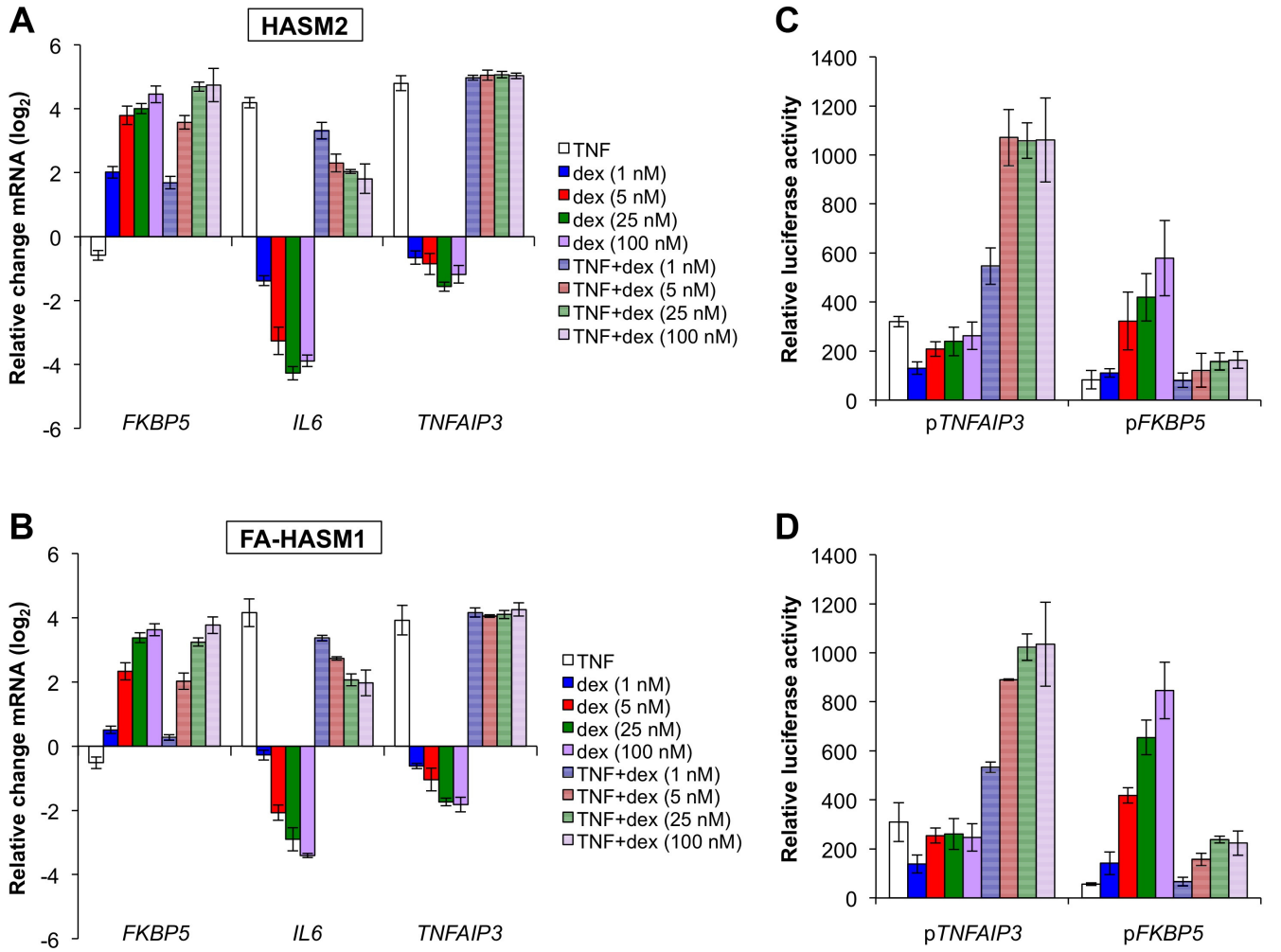

Fig. 4. Dose-response analysis of dex effects on TNF-mediated induction of *TNFAIP3* and cooperation with p65. *A* and *B*: qPCR analysis of *FKPB5*, *IL6*, and *TNFAIP3* expression in HASM2 (*A*) and FA-HASM1 (*B*) cells treated as indicated for 4 h. *C* and *D*: luciferase activity of p*TNFAIP3* and p*FKBP5* reporter constructs transiently transfected into HASM2 (*C*) or FA-HASM1 (*D*) and treated as shown for 8 h. Data are normalized and expressed as described in Figure 3.

TNF-mediated induction of both pro- and anti-inflammatory ASM genes is strongly repressed by TNFAIP3 overexpression. Whereas negative feedback control of NF-κB signaling has been well studied in other cell types, there are no reports on A20/TNFAIP3 function in HASM. Therefore, to explore the effect of TNFAIP3 on NF-κB-driven inflammatory signaling in ASM, we transduced HASM2 cells with adenoviral expression constructs for TNFAIP3 (Ad-*TNFAIP3*) or GFP (Ad-*GFP*) and subsequently exposed cells to 4 h of TNF, dex, or TNF + dex. As presented in Figure 5*A*, TNF-mediated induction of both pro- and anti-inflammatory genes was generally attenuated in cells transduced with Ad-*TNFAIP3* compared with Ad-*GFP*, although some targets exhibited greater sensitivity than others. Furthermore, Ad-*TNFAIP3* appeared to facilitate the repressive effects of dex on proinflammatory gene induction (Fig. 5*A*, *top*). Ad-*TNFAIP3* overexpression was verified using qPCR primers targeting an exonic region (Fig. 5*B*) and at the protein level (Fig. 5*C*). These data are consistent with an anti-inflammatory role for A20/TNFAIP3 in ASM.

TNIP1 overexpression suppresses TNF-induced transcription in ASM. We next asked whether TNIP1, another target of GR–NF-κB cooperation, exhibits anti-inflammatory function in ASM. By a similar approach to that described above, HASM2 cells were transduced with an adenoviral expression construct for TNIP1 (Ad-*TNIP1*) or Ad-*GFP* before treatment with TNF, dex, or TNF + dex for 4 h. Figure 6*A* (*top*) indicates that TNF-mediated induction of a subset of proinflammatory targets (e.g., *IL1B*, *CXCL8*, and *TNF*) was reduced by Ad-*TNIP1*. Interestingly, TNF induction of several genes, including the anti-inflammatory genes *TNFAIP3* and *NFKBIA*, was relatively unaffected by Ad-*TNIP1* (Fig. 6*A*, *bottom*). This is in contrast to Ad-*TNFAIP3* (Fig. 5), which exhibited stronger and less selective inhibitory effects on TNF-mediated gene induction. Ad-*TNIP1* overexpression was confirmed at both the transcript (Fig. 6*B*) and protein (Fig. 6*C*) levels.

Maximal cytokine repression by GCs in ASM requires TNFAIP3. Whereas overexpression data indicate that TNFAIP3 and TNIP1 can suppress transcriptional responses to TNF, these data do not establish whether negative feedback control is required for GR-mediated cytokine repression. Therefore, to examine the role of TNFAIP3 in proinflammatory gene suppression by GCs in ASM, we transfected HASM2 cells with siRNA against *TNFAIP3* (si-*TNFAIP3*) or a nontar-

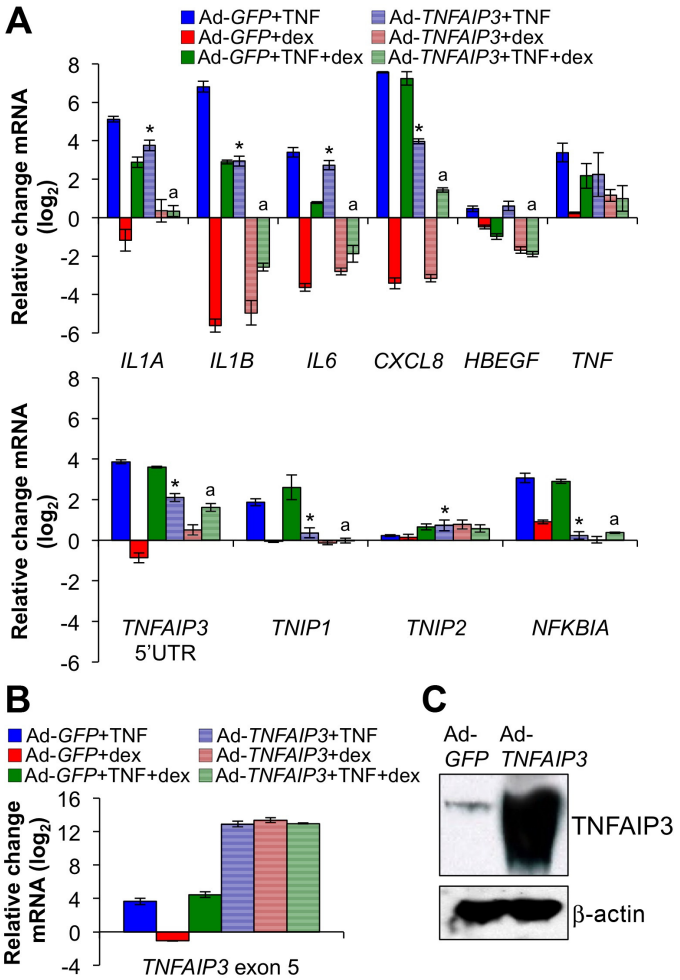

Fig. 5. Adenoviral overexpression of TNFAIP3 potently suppresses TNF-mediated gene induction in ASM. *A*: qPCR analysis of pro- (*top*) and anti-inflammatory (*bottom*) gene expression in HASM2 cells transduced with adenoviral expression constructs for TNFAIP3 (Ad-*TNFAIP3*) or green fluorescent protein (GFP) (Ad-*GFP*) as control ~17 h prior to treatment with TNF, dex, or TNF + dex for 4 h. Bars indicate mean normalized C_T values on a $\log_2$ scale (+SD) relative to Ad-*GFP* + vehicle-treated controls. *$P \leq 0.05$ vs. response of same gene in Ad-*GFP* + TNF-treated cells, [a]$P < 0.05$ vs. response of same gene in Ad-GFP + TNF + dex-treated cells. *B*: qPCR analysis of exogenous *TNFAIP3* expression using primers targeting an exonic region encoded by the Ad-*TNFAIP3* construct in cells described in *A*. *C*: Western blot analysis confirming induction of TNFAIP3 protein expression after 17 h of exposure to Ad-*TNFAIP3*. Beta-actin served as a loading control.

geting control (si-Ctrl) prior to treatment with TNF, dex, or TNF + dex for 24 h. All tested proinflammatory TNF target genes, with the exception of *HBEGF*, exhibited increased induction by TNF and decreased percent repression by dex in si-*TNFAIP3*- vs. si-Ctrl-transfected cells (Fig. 7*A*). We confirmed reduction of TNFAIP3 protein expression by si-*TNFAIP3* in lysates from similarly treated HASM2 cells and further demonstrated reduced dex-repression of IL-1β at the protein level with si-*TNFAIP3* relative to si-Ctrl (Fig. 7*B*). These results indicate that TNFAIP3 is required for maximal repression of proinflammatory gene induction in ASM. Taken together, our data implicate cooperation between GR and p65 at targets such as TNFAIP3 as contributing to steroid-mediated cytokine repression in ASM.

DISCUSSION

This study demonstrates that in cultured HASM, GC signaling results in selective regulation of pro- vs. anti-inflammatory targets of TNF, a cytokine that exhibits increased expression in asthma and chronic obstructive pulmonary disease (40). Using ChIP and reporter assays in HASM, we also demonstrated that GR and NF-κB directly and cooperatively regulate the TNF-induced NF-κB repressor, A20, through an intronic enhancer whose activity is further augmented by β-adrenoceptor agonists. Moreover, our data indicate that A20 and its interacting factor, TNIP1, repress HASM cytokine expression, and that A20 is required for maximal cytokine suppression by GCs in HASM. Thus cooperative induction of A20 by GR and p65 is a mediator of the repressive effects of steroids on HASM cytokine expression. When viewed in the context of our similar

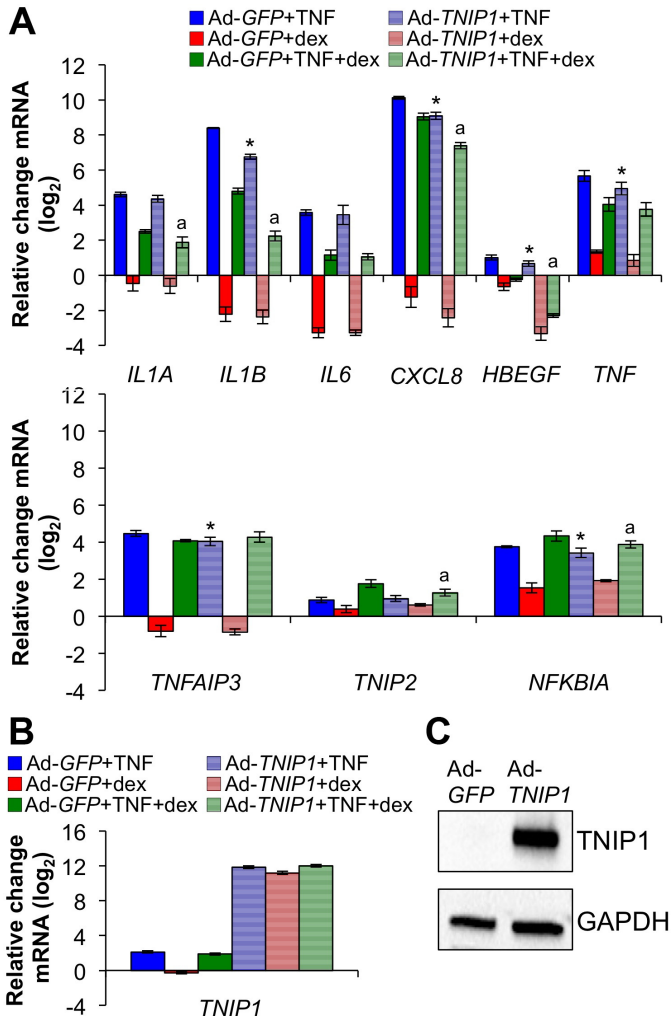

Fig. 6. Ad-*TNIP1* exerts repressive effects on transcriptional responses to TNF. *A*: qPCR analysis of pro- (*top*) and anti-inflammatory (*bottom*) gene expression in HASM2 cells transduced with adenoviral expression constructs for TNIP1 (Ad-*TNIP1*) or Ad-*GFP* control prior to 4 h of treatment with TNF, dex, or TNF + dex. Bars reflect mean normalized C_T values on a $\log_2$ scale (+SD) relative to Ad-GFP + vehicle-treated controls. *$P \leq 0.05$ vs. response of same gene in Ad-*GFP* + TNF-treated cells, [a]$P < 0.05$ vs. response of same gene in Ad-GFP + TNF + dex-treated cells. *B*: analysis of exogenous *TNIP1* mRNA expression in cells treated as in *A*. *C*: verification of TNIP1 protein induction by Western blotting following 17 h of exposure to Ad-*TNIP1*, with GAPDH as a loading control.

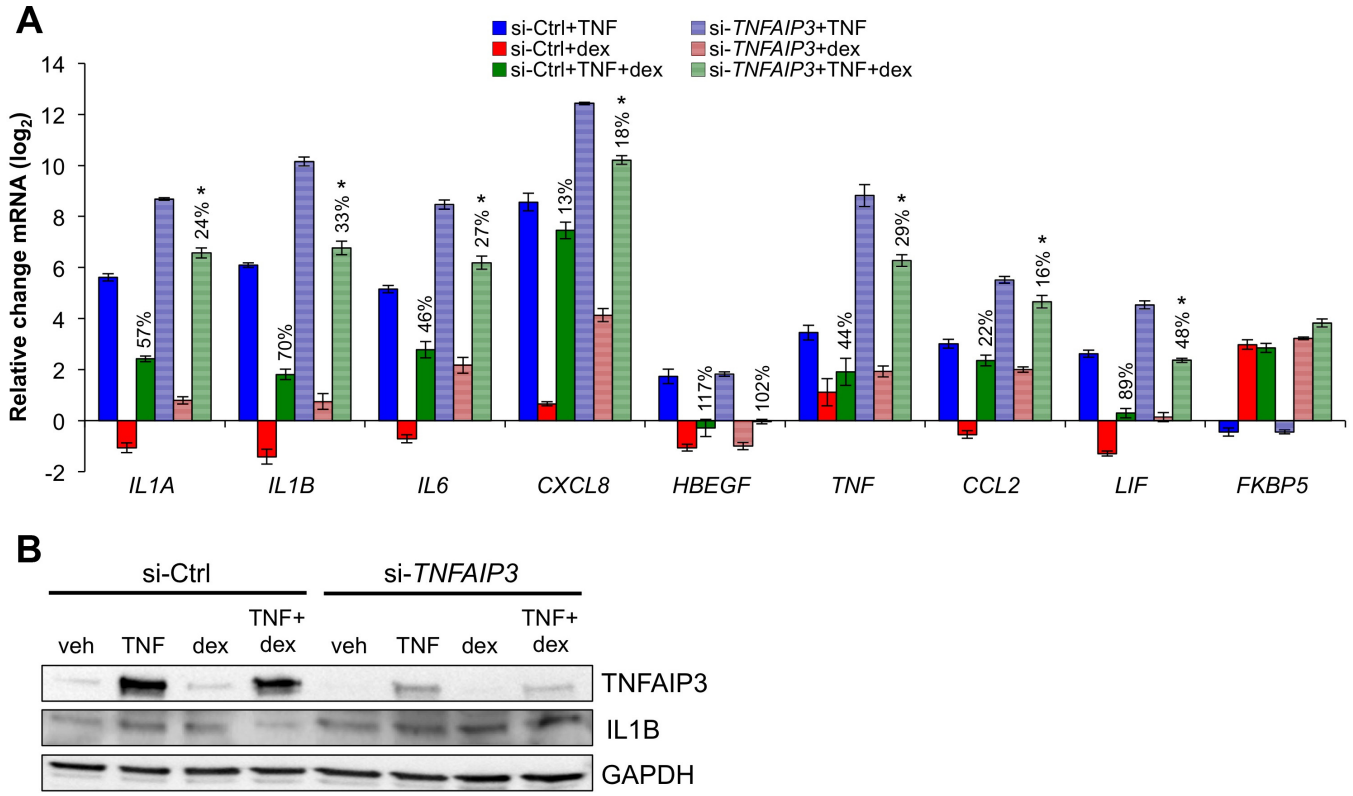

Fig. 7. TNFAIP3 is required for maximal ASM cytokine suppression by glucocorticoids. *A*: HASM2 cells were transfected with small interfering RNA (siRNA) targeting TNFAIP3 (si-*TNFAIP3*) or control (si-Ctrl) and ~48 h later were treated with TNF, dex, or TNF + dex for 24 h. Proinflammatory gene expression was assayed using qPCR. Bars indicate mean normalized C_T values on a $\log_2$ scale (+SD) vs. si-Ctrl + vehicle-treated cells. *$P \leq 0.05$ compared with the response of the same gene to si-Ctrl + TNF + dex-treated cells. Percent repression relative to TNF response within respective siRNA treatment groups is indicated. *B*: Western blots of HASM2 lysates from the experiment described in *A* verifying reduction of TNFAIP3 protein expression with si-*TNFAIP3* and indicating the effect of TNFAIP3 knockdown on basal and induced expression of the proinflammatory cytokine IL-1β. GAPDH served as a loading control.

findings and proposed model in human airway epithelial cells (28), our data provide support for a general paradigm in which GR and NF-κB cooperation contribute mechanistically to inflammatory repression by steroid-based therapies in airway disease.

Numerous studies using cultured HASM have defined important targets through which GCs exert potentially beneficial effects on ASM in asthma (22, 42). These include genes that primarily affect noninflammatory pathways such as *CD38*, an NF-κB target that increases ASM contractility and is repressed by GCs (25, 29, 59), and *RGS2* (66), whose induction by GR is similarly implicated in modulating ASM contraction (25). In addition, a host of genes with presumptive proinflammatory activity such as *IL6*, *CCL2*, and *RANTES*, among others, are potently repressed in ASM by steroid treatment (2, 45). Tethering-based interactions between GR and inflammatory transcription factors such as NF-κB, resulting in GR-mediated recruitment of repressive complexes, are frequently viewed as contributing to transcriptional repression by GCs in HASM (4). However, there is relatively scant mechanistic data in direct support of this contention, and studies unambiguously demonstrating that GR directly recruits putative repressors, such as HDAC2, to inflammatory promoters do not appear to have been performed in ASM. In contrast, several directly induced transcriptional targets of GR (52), most notably DUSP1 (31, 49), have been shown to mediate cytokine repression. Our work extends these prior findings on transcriptional activation by GR and implicates an important role for GC-mediated induction of negative feedback controllers of NF-κB, such as A20, in steroid efficacy. Intriguingly, another GR-induced target that represses cytokine expression in ASM, *CRISPLD2*, is induced by IL-1β in ASM (24), suggesting that it may also function in negative feedback responses to inflammatory stimuli. It will be intriguing in the future to determine the mechanistic basis for GR-p65 cooperative induction of genes such as A20, and the general role of negative feedback controllers of inflammation in mediating steroid responses to diverse inflammatory stimuli.

In our analysis of a limited number of patient-derived ASM samples, although there was a clear distinction between the effects of dex on mRNA levels of pro- and anti-inflammatory targets of TNF across the sample set, we did not observe obvious differences in steroid responses between cultured ASM cell samples, which included two primary cell lines from patients with normal ASM and two from patients with FA. It is nevertheless intriguing to consider our results within the general context of steroid resistance in ASM. Although the term "steroid resistance" is linked to a range of possible meanings in the literature (5, 7, 11, 60), one reasonable molecular definition of steroid resistance is a reduced capacity of ligand-activated GR to regulate transcription. In many cell types, including ASM, cytokines are implicated as causing steroid resistance that corresponds to this definition (52, 60). Compatible with this notion, our data for induction of the *FKBP5* reporter by

GR (Fig. 3) shows that TNF treatment results in reduced dex-mediated activation. However, TNF treatment clearly enhanced dex-mediated induction of the *TNFAIP3* luciferase reporter. Thus the assignment of steroid resistance caused by TNF treatment based on analysis of a single reporter fails to capture the complexity of cross-talk between GR and NF-κB. In addition, although our data clearly establish the importance of A20 in steroid action in ASM, analysis of a broader set of samples from severe asthma is needed to determine whether dysfunctional negative feedback control contributes to reduced steroid sensitivity or elevated basal cytokine expression and secretion in severe asthma. Indeed, it appeared that compared with A20, TNIP1 expression may have been preferentially elevated at the protein level by dex + TNF in the fatal HASM line we examined (Fig. 1, *E* and *F*), and secreted levels of CXCL8 were not reduced by dex at 24 h. These data suggest possible heterogeneity in the negative feedback control of NF-κB in ASM that requires additional investigation.

A number of studies, including our own data on airway epithelial cells (28) and in vivo studies of GR in murine liver (36), have suggested that a subset of cellular GR is localized to the nucleus and can interact with DNA in the absence of supplemental ligand. The data presented here extend on prior findings of "unliganded" GR in ASM to establish that under basal culture conditions, GR interacts directly with regulatory regions in genes such as *TNFAIP3* and *IL6* (see Fig. 2) that exhibit changes in expression upon exposure to exogenous GCs (see Fig. 1). Moreover, aligned with prior ASM studies (8, 26), this process appears to be enhanced at certain loci by TNF. For example, GR occupancy levels were markedly increased by TNF treatment compared with vehicle at several tested loci, including *IL1A* and *CXCL8* (see Fig. 2). It appears, however, that TNF-driven GR occupancy in the absence of supplemental ligand does not efficiently enable the dominant effect of supplemental GC treatment on gene expression at individual loci. Indeed, the addition of dex resulted in repression of *IL1A* and *CXCL8* mRNA expression (Fig. 1), without a substantial change in TNF-induced GR occupancy (Fig. 2). Thus the biologic significance of unliganded GR and the mechanistic basis for GR nuclear localization in the absence of supplemental ligand are not yet clear. Despite these uncertainties, we are confident in the methodology we have applied to determine GR occupancy at these loci, which is based on comparing the test regions to unoccupied negative control regions within the same sample. Applying this approach has led to us generating robust and reproducible ChIP-seq data for GR occupancy in airway epithelial cells (28), including evidence for GR occupancy in the absence of ligand, suggesting that our ChIP-qPCR findings here are unlikely to be artifactual.

The identification of new targets and pathways for steroid action in ASM is important for understanding mechanisms of steroid resistance in severe asthma and developing improved therapies. Our work presented here substantially expands on our understanding of GC action in ASM by defining A20/TNFAIP3 as an induced target of GCs that contributes to steroid-mediated cytokine repression. In the context of work from others implicating A20 in mediating aspects of allergic airway inflammation (56), A20 now emerges as a potential therapeutic target in asthma. More generally, our data also highlight the importance of negative feedback control of inflammation in ASM and suggest that selective cross-talk between GCs and NF-κB may underpin steroid-mediated repression of TNF signaling. In that regard, whereas induction of NF-κB is associated with asthma exacerbations, asthmatic inflammation is heterogeneous and integrates diverse signals from a diverse array of cytokines and cell types (9, 18, 33, 48). It will be of significant future interest to determine whether GCs augment negative feedback mechanisms in the complex inflammatory milieu of the asthmatic airway.

ACKNOWLEDGMENTS

This work was supported by National Heart, Lung, and Blood Institute Grant R01 HL-109557 to A. N. Gerber and by institutional funds from National Jewish Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.N.G. conception and design of research; S.K.S., M.O.A., V.K., and G.C. performed experiments; S.K.S., M.O.A., V.K., and A.N.G. analyzed data; S.K.S., M.O.A., V.K., R.A.P., and A.N.G. interpreted results of experiments; S.K.S. prepared figures; S.K.S. and A.N.G. drafted manuscript; S.K.S., V.K., and A.N.G. edited and revised manuscript; S.K.S., M.O.A., V.K., G.C., R.A.P., and A.N.G. approved final version of manuscript.

REFERENCES

1. **Altonsy MO, Sasse SK, Phang TL, Gerber AN.** Context-dependent cooperation between nuclear factor kappaB (NF-κB) and the glucocorticoid receptor at a TNFAIP3 intronic enhancer: a mechanism to maintain negative feedback control of inflammation. *J Biol Chem* 289: 8231–8239, 2014.
2. **Ammit AJ, Lazaar AL, Irani C, O'Neill GM, Gordon ND, Amrani Y, Penn RB, Panettieri RA Jr.** Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists. *Am J Respir Cell Mol Biol* 26: 465–474, 2002.
3. **Amrani Y, Syed F, Huang C, Li K, Liu V, Jain D, Keslacy S, Sims MW, Baidouri H, Cooper PR, Zhao H, Siddiqui S, Brightling CE, Griswold D, Li L, Panettieri RA Jr.** Expression and activation of the oxytocin receptor in airway smooth muscle cells: Regulation by TNFalpha and IL-13. *Respir Res* 11: 104, 2010.
4. **Barnes PJ.** Biochemical basis of asthma therapy. *J Biol Chem* 286: 32899–32905, 2011.
5. **Barnes PJ.** Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 131: 636–645, 2013.
6. **Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, Consler TG, Parks DJ, Stewart EL, Willson TM, Lambert MH, Moore JT, Pearce KH, Xu HE.** Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. *Cell* 110: 93–105, 2002.
7. **Boardman C, Chachi L, Gavrila A, Keenan CR, Perry MM, Xia YC, Meurs H, Sharma P.** Mechanisms of glucocorticoid action and insensitivity in airways disease. *Pulm Pharmacol Ther* 29: 129–143, 2014.
8. **Bouazza B, Debba-Pavard M, Amrani Y, Isaacs L, O'Connell D, Ahamed S, Formella D, Tliba O.** Basal p38 mitogen-activated protein kinase regulates unliganded glucocorticoid receptor function in airway smooth muscle cells. *Am J Respir Cell Mol Biol* 50: 301–315, 2014.
9. **Bradding P, Arthur G.** Mast cells in asthma — state of the art. *Clin Exp Allergy* 46: 194–263, 2016.
10. **Clark AR, Belvisi MG.** Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor. *Pharmacol Ther* 134: 54–67, 2012.
11. **Clemmer GL, Wu AC, Rosner B, McGeachie MJ, Litonjua AA, Tantisira KG, Weiss ST.** Measuring the corticosteroid responsiveness endophenotype in asthmatic patients. *J Allergy Clin Immunol* 136: 274–281, 2015.
12. **Coornaert B, Carpentier I, Beyaert R.** A20: central gatekeeper in inflammation and immunity. *J Biol Chem* 284: 8217–8221, 2009.
13. **Damera G, Tliba O, Panettieri RA Jr.** Airway smooth muscle as an immunomodulatory cell. *Pulm Pharmacol Ther* 22: 353–359, 2009.

14. **De Bosscher K, Haegeman G, Elewaut D.** Targeting inflammation using selective glucocorticoid receptor modulators. *Curr Opin Pharmacol* 10: 497–504, 2010.
15. **De Bosscher K, Vanden Berghe W, Haegeman G.** The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev* 24: 488–522, 2003.
16. **Dekkers BG, Pehlic A, Mariani R, Bos IS, Meurs H, Zaagsma J.** Glucocorticosteroids and β_2-adrenoceptor agonists synergize to inhibit airway smooth muscle remodeling. *J Pharmacol Exp Ther* 342: 780–787, 2012.
17. **Durrani SR, Viswanathan RK, Busse WW.** What effect does asthma treatment have on airway remodeling? *J Allergy Clin Immunol* 128: 439–448, 2011.
18. **Erle DJ, Sheppard D.** The cell biology of asthma. *J Cell Biol* 205: 621–631, 2014.
19. **Frijters R, Fleuren W, Toonen EJ, Tuckermann JP, Reichardt HM, van der Maaden H, van Elsas A, van Lierop MJ, Dokter W, de Vlieg J, Alkema W.** Prednisolone-induced differential gene expression in mouse liver carrying wild type or a dimerization-defective glucocorticoid receptor. *BMC Genomics* 11: 359, 2010.
20. **Gerber AN, Masuno K, Diamond MI.** Discovery of selective glucocorticoid receptor modulators by multiplexed reporter screening. *Proc Natl Acad Sci USA* 106: 4929–4934, 2009.
21. **Gertz J, Savic D, Varley KE, Partridge EC, Safi A, Jain P, Cooper GM, Reddy TE, Crawford GE, Myers RM.** Distinct properties of cell-type-specific and shared transcription factor binding sites. *Mol Cell* 52: 25–36, 2013.
22. **Hakonarson H, Halapi E, Whelan R, Gulcher J, Stefansson K, Grunstein MM.** Association between IL-1beta/TNF-alpha-induced glucocorticoid-sensitive changes in multiple gene expression and altered responsiveness in airway smooth muscle. *Am J Respir Cell Mol Biol* 25: 761–771, 2001.
23. **Halayko AJ, Tran T, Ji SY, Yamasaki A, Gosens R.** Airway smooth muscle phenotype and function: interactions with current asthma therapies. *Curr Drug Targets* 7: 525–540, 2006.
24. **Himes BE, Jiang X, Wagner P, Hu R, Wang Q, Klanderman B, Whitaker RM, Duan Q, Lasky-Su J, Nikolos C, Jester W, Johnson M, Panettieri RA Jr, Tantisira KG, Weiss ST, Lu Q.** RNA-Seq transcriptome profiling identifies CRISPLD2 as a glucocorticoid responsive gene that modulates cytokine function in airway smooth muscle cells. *PLoS One* 9: e99625, 2014.
25. **Holden NS, Bell MJ, Rider CF, King EM, Gaunt DD, Leigh R, Johnson M, Siderovski DP, Heximer SP, Giembycz MA, Newton R.** β2-Adrenoceptor agonist-induced RGS2 expression is a genomic mechanism of bronchoprotection that is enhanced by glucocorticoids. *Proc Natl Acad Sci USA* 108: 19713–19718, 2011.
26. **Hu A, Josephson MB, Diener BL, Nino G, Xu S, Paranjape C, Orange JS, Grunstein MM.** Pro-asthmatic cytokines regulate unliganded and ligand-dependent glucocorticoid receptor signaling in airway smooth muscle. *PLoS One* 8: e60452, 2013.
27. **John AE, Zhu YM, Brightling CE, Pang L, Knox AJ.** Human airway smooth muscle cells from asthmatic individuals have CXCL8 hypersecretion due to increased NF-kappa B p65, C/EBP beta, and RNA polymerase II binding to the CXCL8 promoter. *J Immunol* 183: 4682–4692, 2009.
28. **Kadiyala V, Sasse SK, Altonsy MO, Berman R, Chu HW, Phang TL, Gerber AN.** Cistrome-based cooperation between airway epithelial glucocorticoid receptor and NF-κB orchestrates anti-inflammatory effects. *J Biol Chem* 291: 12673–12687, 2016.
29. **Kang BN, Jude JA, Panettieri RA Jr, Walseth TF, Kannan MS.** Glucocorticoid regulation of CD38 expression in human airway smooth muscle cells: role of dual specificity phosphatase 1. *Am J Physiol Lung Cell Mol Physiol* 295: L186–L193, 2008.
30. **Kang NI, Yoon HY, Lee YR, Won M, Chung MJ, Park JW, Hur GM, Lee HK, Park BH.** A20 attenuates allergic airway inflammation in mice. *J Immunol* 183: 1488–1495, 2009.
31. **King EM, Holden NS, Gong W, Rider CF, Newton R.** Inhibition of NF-κB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. *J Biol Chem* 284: 26803–26815, 2009.
32. **Kleiman A, Hübner S, Rodriguez Parkitna JM, Neumann A, Hofer S, Weigand MA, Bauer M, Schmid W, Schütz G, Libert C, Reichardt HM, Tuckermann JP.** Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. *FASEB J* 26: 722–729, 2012.
33. **KleinJan A.** Airway inflammation in asthma: key players beyond the Th2 pathway. *Curr Opin Pulm Med* 22: 46–52, 2016.
34. **Knox AJ.** The scientific rationale of combining inhaled glucocorticoids and long acting beta 2 adrenoceptor agonists. *Curr Pharm Des* 8: 1863–1869, 2002.
35. **Lauzon AM, Martin JG.** Airway hyperresponsiveness; smooth muscle as the principal actor. *F1000Res* Mar 9: 5, 2016.
36. **Lim HW, Uhlenhaut NH, Rauch A, Weiner J, Hübner S, Hübner N, Won KJ, Lazar MA, Tuckermann J, Steger DJ.** Genomic redistribution of GR monomers and dimers mediates transcriptional response to exogenous glucocorticoid in vivo. *Genome Res* 25: 836–844, 2015.
37. **Lu X, Malumbres R, Shields B, Jiang X, Sarosiek KA, Natkunam Y, Tiganis T, Lossos IS.** PTP1B is a negative regulator of interleukin 4-induced STAT6 signaling. *Blood* 112: 4098–4108, 2008.
38. **Ma A, Malynn BA.** A20: linking a complex regulator of ubiquitylation to immunity and human disease. *Nat Rev Immunol* 12: 774–785, 2012.
39. **Masuno K, Haldar SM, Jeyaraj D, Mailloux CM, Huang X, Panettieri RA Jr, Jain MK, Gerber AN.** Expression profiling identifies Klf15 as a glucocorticoid target that regulates airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 45: 642–649, 2011.
40. **Matera MG, Calzetta L, Cazzola M.** TNF-alpha inhibitors in asthma and COPD: we must not throw the baby out with the bath water. *Pulm Pharmacol Ther* 23: 121–128, 2010.
41. **Meijsing SH.** Mechanisms of glucocorticoid-regulated gene transcription. *Adv Exp Med Biol* 872: 59–81, 2015.
42. **Misior AM, Deshpande DA, Loza MJ, Pascual RM, Hipp JD, Penn RB.** Glucocorticoid- and protein kinase A-dependent transcriptome regulation in airway smooth muscle. *Am J Respir Cell Mol Biol* 41: 24–39, 2009.
43. **Miyata M, Lee JY, Susuki-Miyata S, Wang WY, Xu H, Kai H, Kobayashi KS, Flavell RA, Li JD.** Glucocorticoids suppress inflammation via the upregulation of negative regulator IRAK-M. *Nat Commun* 6: 6062, 2015.
44. **Panettieri RA Jr, Kotlikoff MI, Gerthoffer WT, Hershenson MB, Woodruff PG, Hall IP, Banks-Schlegel S.** Airway smooth muscle in bronchial tone, inflammation, and remodeling: basic knowledge to clinical relevance. *Am J Respir Crit Care Med* 177: 248–252, 2008.
45. **Patel JK, Clifford RL, Deacon K, Knox AJ.** Ciclesonide inhibits TNFα- and IL-1β-induced monocyte chemotactic protein-1 (MCP-1/CCL2) secretion from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 302: L785–L792, 2012.
46. **Patel M, Shaw D.** A review of standard pharmacological therapy for adult asthma — steps 1 to 5. *Chron Respir Dis* 12: 165–176, 2015.
47. **Prakash YS.** Airway smooth muscle in airway reactivity and remodeling: what have we learned? *Am J Physiol Lung Cell Mol Physiol* 305: L912–L933, 2013.
48. **Prakash YS, Martin RJ.** Brain-derived neurotrophic factor in the airways. *Pharmacol Ther* 143: 74–86, 2014.
49. **Quante T, Ng YC, Ramsay EE, Henness S, Allen JC, Parmentier J, Ge Q, Ammit AJ.** Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. *Am J Respir Cell Mol Biol* 39: 208–217, 2008.
50. **Rao NA, McCalman MT, Moulos P, Francoijs KJ, Chatziioannou A, Kolisis FN, Alexis MN, Mitsiou DJ, Stunnenberg HG.** Coactivation of GR and NFKB alters the repertoire of their binding sites and target genes. *Genome Res* 21: 1404–1416, 2011.
51. **Renner F, Schmitz ML.** Autoregulatory feedback loops terminating the NF-kappaB response. *Trends Biochem Sci* 34: 128–135, 2009.
52. **Rider CF, Shah S, Miller-Larsson A, Giembycz MA, Newton R.** Cytokine-induced loss of glucocorticoid function: effect of kinase inhibitors, long-acting beta$_2$-adrenoceptor [corrected] agonist and glucocorticoid receptor ligands. *PLoS One* 10: e0116773, 2015.
53. **Rogatsky I, Luecke HF, Leitman DC, Yamamoto KR.** Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc Natl Acad Sci USA* 99: 16701–16706, 2002.
54. **Rogatsky I, Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq CM, Darimont BD, Garabedian MJ, Yamamoto KR.** Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. *Proc Natl Acad Sci USA* 100: 13845–13850, 2003.
55. **Sasse SK, Mailloux CM, Barczak AJ, Wang Q, Altonsy MO, Jain MK, Haldar SM, Gerber AN.** The glucocorticoid receptor and KLF15 regulate

gene expression dynamics and integrate signals through feed-forward circuitry. *Mol Cell Biol* 33: 2104–2115, 2013.

56. **Schuijs MJ, Willart MA, Vergote K, Gras D, Deswarte K, Ege MJ, Madeira FB, Beyaert R, van Loo G, Bracher F, von Mutius E, Chanez P, Lambrecht BN, Hammad H.** Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. *Science* 349: 1106–1110, 2015.
57. **Stahn C, Lowenberg M, Hommes DW, Buttgereit F.** Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. *Mol Cell Endocrinol* 275: 71–78, 2007.
58. **Stewart AG, Fernandes D, Tomlinson PR.** The effect of glucocorticoids on proliferation of human cultured airway smooth muscle. *Br J Pharmacol* 116: 3219–3226, 1995.
59. **Tirumurugaan KG, Kang BN, Panettieri RA, Foster DN, Walseth TF, Kannan MS.** Regulation of the cd38 promoter in human airway smooth muscle cells by TNF-alpha and dexamethasone. *Respir Res* 9: 26, 2008.
60. **Tliba O, Damera G, Banerjee A, Gu S, Baidouri H, Keslacy S, Amrani Y.** Cytokines induce an early steroid resistance in airway smooth muscle cells: novel role of interferon regulatory factor-1. *Am J Respir Cell Mol Biol* 38: 463–472, 2008.
61. **Vandevyver S, Dejager L, Van Bogaert T, Kleyman A, Liu Y, Tuckermann J, Libert C.** Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation. *J Clin Invest* 122: 2130–2140, 2012.
62. **Verstrepen L, Carpentier I, Beyaert R.** The biology of A20-binding inhibitors of NF-kappaB activation (ABINs). *Adv Exp Med Biol* 809: 13–31, 2014.
63. **Vettorazzi S, Bode C, Dejager L, Frappart L, Shelest E, Klaβen C, Tasdogan A, Reichardt HM, Libert C, Schneider M, Weih F, Henriette Uhlenhaut N, David JP, Gräler M, Kleiman A, Tuckermann JP.** Glucocorticoids limit acute lung inflammation in concert with inflammatory stimuli by induction of SphK1. *Nat Commun* 6: 7796, 2015.
64. **Werner SL, Kearns JD, Zadorozhnaya V, Lynch C, O'Dea E, Boldin MP, Ma A, Baltimore D, Hoffmann A.** Encoding NF-kappaB temporal control in response to TNF: distinct roles for the negative regulators IkappaBalpha and A20. *Genes Dev* 22: 2093–2101, 2008.
65. **Wertz I, Dixit V.** A20–a bipartite ubiquitin editing enzyme with immunoregulatory potential. *Adv Exp Med Biol* 809: 1–12, 2014.
66. **Xie Y, Jiang H, Nguyen H, Jia S, Berro A, Panettieri RA Jr, Wolff DW, Abel PW, Casale TB, Tu Y.** Regulator of G protein signaling 2 is a key modulator of airway hyperresponsiveness. *J Allergy Clin Immunol* 130: 968–976, 2012.
67. **Yasunaga S, Yuyama N, Arima K, Tanaka H, Toda S, Maeda M, Matsui K, Goda C, Yang Q, Sugita Y, Nagai H, Izuhara K.** The negative-feedback regulation of the IL-13 signal by the IL-13 receptor alpha2 chain in bronchial epithelial cells. *Cytokine* 24: 293–303, 2003.