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Context-dependent Cooperation between Nuclear Factor κB (NF-κB) and the Glucocorticoid Receptor at a *TNFAIP3* Intronic Enhancer

A MECHANISM TO MAINTAIN NEGATIVE FEEDBACK CONTROL OF INFLAMMATION[*S]

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Background: The effects of glucocorticoids on the expression of negative feedback regulators of NF-κB are not well understood.
Results: A novel intronic enhancer for *TNFAIP3* was synergistically induced by the glucocorticoid receptor and NF-κB.
Conclusion: The glucocorticoid receptor can cooperate with NF-κB to enhance the expression of anti-inflammatory genes such as *TNFAIP3*.
Significance: These results establish a novel mechanism for anti-inflammatory effects of glucocorticoids.

TNF expression is elevated in asthma and other inflammatory airway diseases that are commonly treated with glucocorticoid-based therapies, but the impact of glucocorticoids on negative feedback control of TNF is not well understood. We analyzed the effect of dexamethasone, a potent synthetic glucocorticoid, on TNF-regulated gene expression in cultured airway epithelial cells. Although dexamethasone-mediated activation of the glucocorticoid receptor (GR) potently repressed expression of *IL1β*, *IL8*, and several other pro-inflammatory TNF targets, the expression of anti-inflammatory TNF targets such as *TNFAIP3* (*A20*) and *NFKBIA* was selectively spared or augmented by dexamethasone treatment. Despite divergent effects on gene expression, GR and NF-κB occupancy at the *TNFAIP3* locus and GR-repressed targets was similar. A co-occupied intronic *TNFAIP3* regulatory element mediated cooperative enhancement of transcription by GR and NF-κB that required the presence of a functional GR binding site (GBS). GBS exchanges between reporters for *TNFAIP3* and *FKBP5*, a canonical GR-induced target, revealed substantial latitude in the GBS sequence requirements for GR/NF-κB cooperation, suggesting that the *TNFAIP3* GBS acts primarily as a docking site in this context. Supporting this notion, a selective GR ligand with only weak agonist activity for induction of *FKBP5* enabled robust GR/NF-κB cooperative induction of a mutant *TNFAIP3* reporter harboring the *FKBP5* GBS. Taken together, our data support a model in which the expression of anti-inflammatory targets of TNF is maintained during treatment with glucocorticoids through context-dependent cooperation between GR and NF-κB.

Synthetic glucocorticoids continue to be a mainstay in treating immune-mediated disease (1). Glucocorticoids function primarily through binding to the glucocorticoid receptor (GR),[2] which, in response to ligand, translocates to the nucleus and regulates gene expression, leading to both therapeutic inflammatory suppression and the development of deleterious side effects (1, 2). GR utilizes specific glucocorticoid binding sites and tethering interactions with other transcription factors to associate with DNA, leading to the assembly of activating or repressive complexes and alterations in polymerase II occupancy and processivity (3). GR-mediated gene regulation is itself subject to a wide range of regulatory mechanisms including alternate splicing and posttranslational modification of GR (4–6), restricted expression of co-regulators (7), cell type-specific chromatin architecture (8), and binding site-mediated effects on GR recruitment and activity (9, 10). Despite our growing understanding of the molecular basis for GR function, the precise targets and mechanisms through which GR orchestrates the resolution of inflammation across diverse cellular and disease contexts remain poorly understood.

It has recently become clear that normal termination of inflammatory responses requires the activity of negative feedback circuitry (11, 12). For example, in addition to provoking inflammation, tumor necrosis factor-α (TNF) and other cytokines induce potent anti-inflammatory genes such as *TNFAIP3* (*A20*), a key inhibitor of NF-κB whose dysfunction is associated with inflammatory disorders ranging from rheumatoid arthritis to sepsis (13, 14). Repression of NF-κB function by GR has long been implicated as a crucial determinant in glucocorticoid-based therapeutics, but this activity has generally been attributed to GR associating directly with NF-κB to reduce the expression of specific pro-inflammatory targets (15, 16). Recent studies have suggested greater complexity to GR/NF-κB cross-talk (17–20), with co-occupancy by both factors implicated in driving a variety of effects on steady state target gene expression

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[2] The abbreviations used are: GR, glucocorticoid receptor; GBS, glucocorticoid binding site; dex, dexamethasone; qPCR, quantitative PCR; Ad, adenovirus.

(21, 22). However, these studies have not directly addressed the role of GR in regulating the expression of negative feedback targets of NF-κB, such as *TNFAIP3*, nor have the mechanisms underpinning differential transcriptional consequences of GR/NF-κB cross-talk at specific loci been fully determined.

Elevated expression of TNF occurs in asthma, an inflammatory disorder of the airway that is frequently treated with glucocorticoid-based therapies (23). We therefore assayed expression of pro- and anti-inflammatory targets of TNF in airway epithelial cells after treatment with dexamethasone (dex), a potent glucocorticoid. We applied chromatin immunoprecipitation, reporter assays, binding site swaps, and varied GR ligand chemistries to probe the molecular basis for selectively maintained expression of negative feedback targets of TNF after dex treatment, with a primary focus on *TNFAIP3* regulation. Our results suggest that context-dependent cooperation between GR and NF-κB enables glucocorticoids to preserve negative feedback control of inflammation, thus contributing to the potent effects of glucocorticoids in treating inflammatory disorders of the airway.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Beas-2B cells (ATCC CRL-9609) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose, L-glutamine and supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; HyClone). dex (D1756) was purchased from Sigma and used at a concentration of 100 nM. TNF was purchased from Sigma (T6674) and Life Technologies (PHC3015L). TNF-neutralizing antibody (D1B4) was obtained from Cell Signaling Technology. MK-5932 was previously described (24) and was a generous gift from Merck and Co. Antibodies used for Western analyses were: anti-TNFAIP3 (ab13597), anti-HBEGF (ab92620), anti-β-actin (ab75186) from Abcam; anti-GR (H-300; sc-8992) and anti-NF-κB p65 (C-20; sc-372) from Santa Cruz Biotechnology; and enhanced chemiluminescence (ECL) donkey anti-rabbit IgG-horseradish peroxidase (HRP; NA9340V) and ECL sheep anti-mouse IgG-HRP (NA931V) from GE Healthcare. Antibodies used for chromatin immunoprecipitation (ChIP) were anti-GR (N-499, a generous gift from Dr. Keith Yamamoto, and 1A1, a generous gift from Dr. Miles Pufall) and anti-NFkB p65 (C-20; sc-372) from Santa Cruz Biotechnology. The GFP (Ad-GFP) and *TNFAIP3* (Ad-*TNFAIP3*) adenoviruses were obtained from Welgen; Ad-*TNFAIP3* was constructed using a previously described *TNFAIP3* expression vector (25). siRNA studies were conducted using ON-TARGETplus SMARTpool against human GR (siNR3C1; NM-001020825), human NFkB (siRELA; L-003533-00-0020), and nontargeting control (siCtrl; D-001810-10-05) from Dharmacon.

Plasmids—To generate pTNFAIP3I2, PCR primers to amplify +5670–6491 of the *TNFAIP3* locus were designed based on visualizing published ChIP-seq peaks in the UCSC genome browser and identifying putative GR and NF-κB binding sites using MatInspector (Genomatix). A core binding sequence similarity of 95% was used as a cut-off to identify the consensus GR and NF-κB binding sequences demarcated in Fig. 4. Amplified PCR product was TA-cloned into the pCR 2.1-TOPO vector according to the manufacturer's protocol (Life Technologies) and subsequently introduced into the PGL3 promoter vector (Promega) as a KpnI/XhoI fragment to generate a luciferase reporter for *TNFAIP3I2* enhancer activity. Site-directed mutagenesis of the putative GR binding site was accomplished using the QuikChange II site-directed mutagenesis kit from Agilent Technologies. Primer sequences are listed in the supplemental tables. pFKBP5, pIL8, and p3XNF-κB have been previously described (28, 31, 32) as indicated under "Results."

Transfections, Luciferase Assays, and qPCR—For luciferase assays, cells were plated in 250 μl of antibiotic-free DMEM supplemented with 10% FBS in 48-well plates in at a density of ~4×10^4 cells/well and incubated overnight prior to plasmid transfection. The next day, a complex of Lipofectamine 2000 (1 μl) and total DNA (400 ng) diluted in 50 μl/well Opti-MEM (Life Technologies) was added to each well. DNA complexes were formed from firefly luciferase plasmids and the *Renilla* luciferase (RL) expression vector, pSV40-RL (Promega), at a ratio of 10:1 using Lipofectamine 2000 (Life Technologies) transfection reagent according to the manufacturer's protocol. 18 h after transfection, cells were treated with TNF and/or dex for 8 h. Cells were subsequently assayed for luciferase activity as described previously (26) in biologic quadruplicate. *p* values indicated in the figure legends were calculated using Student's *t* tests or nonparametric analysis. For siRNA transfection, Beas-2B cells were transfected with 25 nM siGR, siNFkB, or siCtrl using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's protocol (Life Technologies). 24–48 h later cells were lysed and assayed for GR or NFkB knockdown using Western blot analysis. Cells were also co-transfected with the above siRNAs in combination with firefly and *Renilla* luciferase plasmids. 24 h later cells were treated as described above and subsequently assayed for luciferase activity. For gene expression analysis, cells were plated in 6-well plates at a density of 3×10^5 cells/well. The next day, cells were treated with TNF and/or dex, and RNA was subsequently prepared using TRIzol reagent and the Pure Link RNA mini kit, both from Life Technologies. RNA was reverse-transcribed, qPCR was performed, and gene expression was quantified as described previously (26). Primer sequences are in the supplemental tables. For adenoviral transduction, Beas 2B cells were transduced with adeno-*TNFAIP3* (Ad-*TNFAIP3*) or control adeno-green fluorescent protein (Ad-GFP) at a multiplicity of infection of 100. After ~17 h cells were treated as indicated under "Results" and assayed for gene expression by quantitative RT-PCR.

Western Analysis—To measure the expression of TNFAIP3 and HBEGF proteins or to assay for GR and NF-κB knockdown at the protein level upon siRNA transfection, treated Beas-2B cells were lysed with radioimmunoprecipitation assay buffer containing 1× protease inhibitor cocktail (Thermo Scientific). 50 μg of protein of each sample were separated by SDS-PAGE and transferred into PVDF membrane (Amersham Biosciences). Membranes were then immunoprobed for the detection of the corresponding proteins. Band visualization was carried out using ECL Plus Western blotting detection system (GE Healthcare).

ChIP—Beas-2B cells were grown to confluence in 100-mm dishes and treated with vehicle (ethanol), 100 nM dex, 20 ng/ml TNF or with a combination of dex and TNF for 1 h. 16% methanol-free formaldehyde (Thermo Scientific) was added directly to the culture medium to a final concentration of 1%, and ChIP was subsequently performed as described (26). Resulting DNA was analyzed using qPCR. Relative occupancy was calculated on a $\log_2$ scale based on comparison with the geometric mean of C_T values for two or three negative control regions. Validity of negative controls was established through demonstrating that amplification of dilute input DNA generated similar C_T values for primers to control and putative occupied regions. Primer sequences are in the supplemental tables.

RESULTS

Glucocorticoids Selectively Spare the Expression of Anti-inflammatory Targets of TNF—TNF is implicated as a driver in inflammatory airway diseases that are treated with glucocorticoids, but the effects of glucocorticoids on negative feedback control of TNF signaling have not been evaluated in airway epithelial cells. We therefore used Beas-2B cells, a human airway epithelial cell line, to analyze the expression of a set of well described pro- and anti-inflammatory targets of TNF signaling. Cells were treated with TNF, dex, which is a potent GR agonist, or a combination of both agents for a total of 4 h. As expected, the expression of several typical pro-inflammatory targets of TNF, such as *IL8* and *HBEGF*, was strongly induced by TNF (Fig. 1*A*, *red bars*). The inductive effect of TNF on each of these genes was abrogated significantly ($p < 0.05$) by co-treatment with dex (Fig. 1*A*, *yellow bars*). In contrast, but concordant with findings from recent studies in other cell types (17, 19, 22), the expression of anti-inflammatory targets of TNF such as *TNFAIP3* (*A20*), *TNIP1*, and *NFKBIA* was spared or augmented by TNF + dex co-treatment in comparison with treatment with TNF alone (Fig. 1*B*, compare *red* and *yellow bars*). The differential effects of dex on the expression of pro- and anti-inflammatory TNF target genes were confirmed at the protein level for HBEGF and TNFAIP3 (Fig. 1*C*). Thus, induction of GR signaling by dex spares or augments the expression of anti-inflammatory targets of TNF, whereas it potently represses the expression of inflammatory mediators.

Next we asked whether differential regulation of pro- and anti-inflammatory gene expression is specific to GR-based inhibition of TNF signaling. We took two approaches to address this question. First, we used a TNF-neutralizing antibody to block upstream TNF signaling. We incubated increasing concentrations of TNF-neutralizing antibody with recombinant TNF for 2 h and treated cells with the resultant complexes for 4 h prior to analyzing gene expression via qPCR. In contrast to the effects of dex treatment, antibody-based blockade of TNF repressed the induction of both pro-inflammatory and anti-inflammatory genes by TNF in a dose-dependent manner (Fig. 2*A*). Second, we used an adenoviral system to overexpress *TNFAIP3* (Ad-*TNFAIP3*) as a mechanism to abrogate TNF signaling through reducing NF-κB activity. Here, gene regulation by TNF, dex, or TNF + dex was analyzed after infection with Ad-*TNFAIP3* in comparison with infection with a control adenovirus (Ad-GFP). Although there were some differences in sensitivity of different targets, forced *TNFAIP3* expression generally reduced the expression of both pro-inflammatory and anti-inflammatory targets (Fig. 2*B*). Primers that amplify a region of the *TNFAIP3* 5′-UTR were used in this experiment to measure endogenous *TNFAIP3* expression (Fig. 2*B*), whereas overexpression of *TNFAIP3* driven by the adenovirus was confirmed both by qPCR using primers for *TNFAIP3* exon 5 (Fig. 2*B*) and by Western blot (Fig. 2*C*). Taken together, these data indicate that, unlike the selective regulatory effects of GR activation, restraining TNF signaling through either upstream blockade or inhibition of NF-κB activity decreases both pro-inflammatory and anti-inflammatory gene expression.

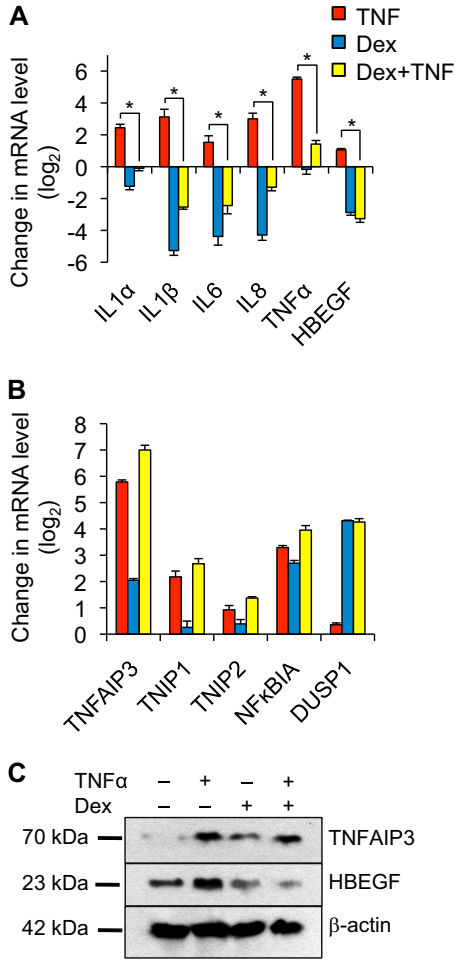

FIGURE 1. **Glucocorticoids selectively repress pro-inflammatory responses to TNF.** *A* and *B*, qPCR analysis of pro-inflammatory (*A*) and anti-inflammatory (*B*) gene expression in Beas-2B cells treated for 4 h with vehicle, TNF (20 ng/ml), dex (100 nM), or TNF + dex as indicated. For both *A* and *B*, bar graphs indicate mean normalized C_T values of biological quadruplicate samples relative to vehicle control + S.D., * indicates $p \leq 0.05$ for relevant comparisons. *C*, Western analysis of TNFAIP3, HBEGF, and β-actin (loading control) proteins using lysates prepared from Beas-2B cells treated as indicated for 24 h.

Patterns of GR and NF-κB Occupancy Are Similar at Pro- and Anti-inflammatory Regulatory Loci—GR is generally believed to mediate therapeutic effects via transrepression of inflammatory transcription factors such as NF-κB and AP-1, whereas gene induction by GR has been associated with side effects (27). However, our gene expression analysis suggested that GR actively regulates the expression of anti-inflammatory genes

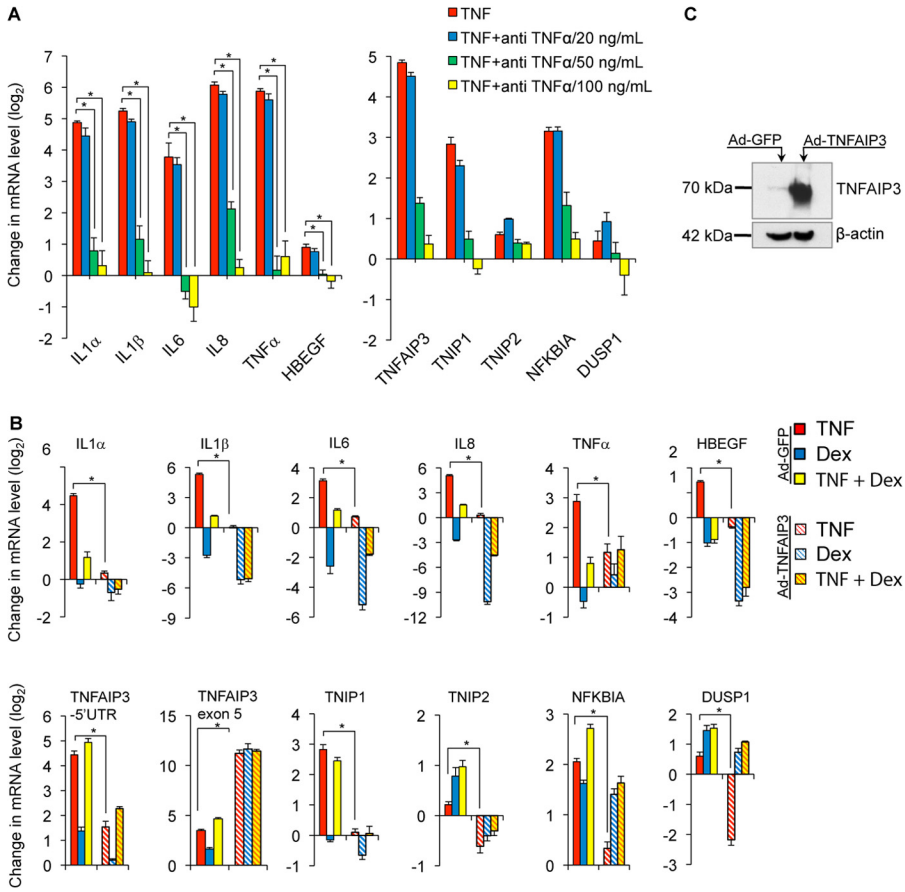

FIGURE 2. **TNF blockade and *TNFAIP3* overexpression repress both pro-inflammatory and anti-inflammatory gene expression in Beas-2B cells.** *A*, relative mRNA levels of pro-inflammatory (*left*) and anti-inflammatory (*right*) genes in Beas-2B cells treated with a combination of TNF (20 ng/ml) and increasing concentrations of anti-TNF-neutralizing antibody for 4 h; TNF was allowed to complex with antibody for 2 h prior to addition to the cells. *B*, effect of adenoviral mediated *TNFAIP3* (Ad-*TNFAIP3*) overexpression on mRNA levels relative to Ad-GFP control of the indicated pro-inflammatory (*top*) and anti-inflammatory (*bottom*) genes in Beas-2B cells treated with TNF (20 ng/ml), dex (100 nM), or both as indicated. For both *A* and *B*, bar graphs indicate mean normalized C_T values of biological quadruplicate samples relative to vehicle-treated control + S.D., * indicates $p \leq 0.05$ for relevant comparisons.

that are induced by TNF. To explore the mechanistic basis for regulation of TNF responses by GR, we applied ChIP to compare GR and NF-κB occupancy at selected pro- and anti-inflammatory loci. For this analysis, factor binding peaks from a published ChIP-seq study of GR and NF-κB occupancy in HeLa cells (22), visualized in the UCSC genome browser, were used to identify putative GR and NF-κB binding regions in Beas-2B cells within the *TNFAIP3*, *NFKBIA*, *IL8*, and *IL1β* loci; a well characterized GR binding site within *FKBP5* was used as a control (28, 29). Occupancy for GR and the p65 subunit of NF-κB was determined relative to non-occupied control regions after exposure to each of four conditions for 1 h: vehicle (ethanol), TNF (20 ng/ml), dex (100 nM), or TNF + dex co-treatment. Occupancy data for both factors in each condition at the interrogated regions are shown in composite in Fig. 3 on a $\log_2$ scale. Even with vehicle treatment, both factors exhibited a significant ($p < 0.05$ and greater than 2-fold) increase in occupancy at each of the interrogated regions (with the exception of NF-κB occupancy of the *TNFAIP3* promoter) in comparison with occupancy of control sites, likely reflecting basal activity of both GR and NF-κB signaling in Beas-2B cells under standard culture conditions. Dex treatment significantly increased GR binding above baseline at tested regions associated with *FKBP5*, *IL8*, *TNFAIP3* (+5936 – 6047), and *NFKBIA*, whereas basal binding at IL1β for GR was increased by TNF treatment, consistent with studies indicating that inflammatory signals can modulate GR localization independent of ligand (30). Across treatment conditions, GR exhibited similar occupancy patterns at the *TNFAIP3* and *IL8* loci despite the dichotomous effects of GR activation on *TNFAIP3* and *IL8* gene expression. Likewise, NF-κB (p65) occupancy at the *TNFAIP3* +5.5 region and the *IL8* promoter was similar across treatment conditions. In aggregate, these data indicate that occupancy of GR and NF-κB in Beas-2B cells does not correlate with a specific transcriptional outcome of the associated target genes.

A Novel Enhancer in the TNFAIP3 Locus Mediates Transcriptional Synergism between GR and NF-κB—Although our ChIP data demonstrate that GR and NF-κB occupancy at binding regions within repressed and induced genes is similar, these results do not directly establish the transcriptional consequences of factor occupancy within these regions. To explore this question, we designed primers to amplify a region in the second intron of *TNFAIP3* that was occupied by both GR and NF-κB (Fig. 4*A*). This specific region was selected for further analysis because it contains a strong consensus GR binding sequence and two consensus NF-κB binding sequences, all three of which are highly conserved across most mammalian species, as well as in lizard and chicken (supplemental Fig. S1).

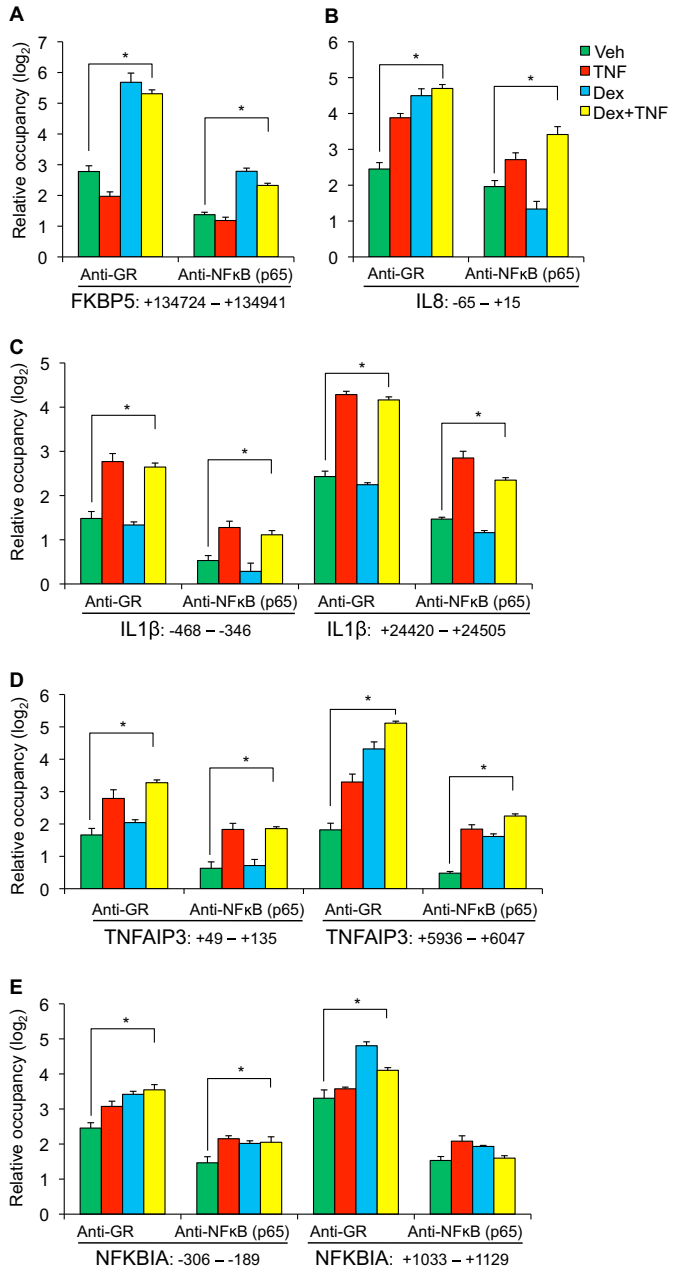

FIGURE 3. **GR and NF-κB occupancy patterns do not distinguish between pro- and anti-inflammatory targets of TNF.** *A–E*, ChIP-qPCR analysis of GR and NFkB (p65) occupancy within the FKBP5 (*A*), *IL8* (*B*), *IL1β* (*C*), *TNFAIP3* (*D*), and *NFKBIA* (*E*) loci in Beas-2B cells treated with TNF (20 ng/ml), dex (100 nM), or both for 1 h. Regions that were interrogated for factor occupancy are identified in relationship to the transcriptional start site of the associated gene, as indicated below the *x* axes. Relative factor occupancy was calculated as a difference between C_T values for each target as compared with the geometric mean of C_T values of three control regions that are not occupied by either GR or NFkB (p65). ChIP experiments were conducted in biological quadruplicate, bars indicate means + S.D. * indicates $p \leq 0.05$ for relevant comparisons. *Veh*, vehicle.

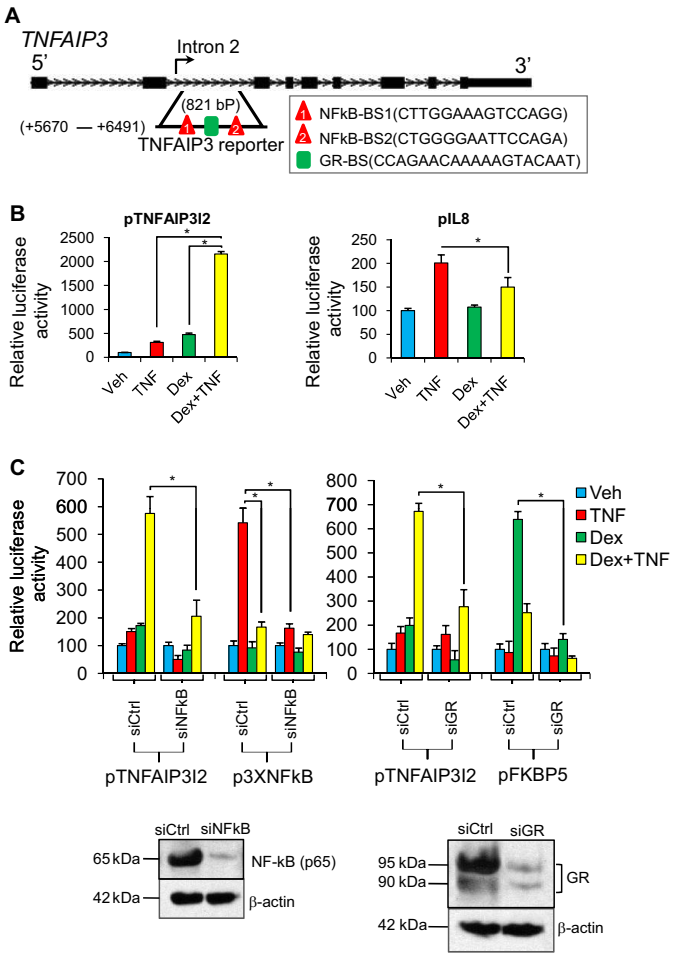

FIGURE 4. **Synergistic induction of an intronic TNFAIP3 enhancer by NF-κB and GR.** *A*, schematic diagram of the TNFAIP3 locus. The region from intron 2 used to generate the pTNFAIP3I2 reporter plasmid and the relative location of strong consensus GR (*GR-BS*) and NF-κB (*NFkB-BS*) binding sites are noted. *B*, relative luciferase activity of the indicated reporter constructs after transfection into Beas-2B cells and treatment with vehicle (*Veh*), TNF, dex, or TNF + dex for 8 h. *C*, relative luciferase activity of pTNFAIP3I2, p3xNFκB (NF-κB control reporter), and pFKBP5 (GR control reporter) in Beas-2B cells co-transfected with control nontargeting siRNA (*siCtrl*), NF-κB-targeting siRNA (*siNFkB*), or GR-targeting siRNA (*siGR*) and treated as indicated. For *B* and *C*, luciferase activity values for each reporter were normalized to a pSV40-*Renilla* control. Bars indicate means of biological quadruplicate + S.D. * indicates $p \leq 0.05$ for relevant comparisons. *D*, Western blot analysis of NFkB, GR, and β-actin protein expression in Beas-2B cells transfected for 24 h with siCtrl, siNFkB, or siGR.

We introduced a roughly 900-bp fragment spanning these binding sequences and conserved flanking sequence into the PGL3 promoter vector to generate a firefly luciferase reporter, pTNAFIP3I2. We transfected pTNFAIP3I2 into Beas-2B cells and subsequently treated cells with vehicle (ethanol), TNF (20 ng/ml), dex (100 nM), or TNF + dex co-treatment for 8 h. We found that pTNFAIP3I2 was induced by both TNF (3.1 ± 0.02) and dex (4.7 ± 0.03-fold) as compared with vehicle treatment. Remarkably, TNF + dex treatment led to robust induction (21.5 ± 0.53-fold) relative to vehicle, which was greater than the product of the individual inductive effects of dex and TNF on pTNFAIP3I2 activity. In contrast, as reported previously (31), TNF-mediated induction of an IL8 reporter, pIL8, which encompasses the IL8 GR/NF-κB binding region we assayed by ChIP, was significantly reduced by treatment with dex (Fig. 4*B*). Thus, dex and TNF induce dichotomous effects on transcription driven from regulatory elements within the *IL8* and *TNFAIP3* loci despite similar occupancy of the corresponding genomic regions by GR and NF-κB.

To confirm that cooperative induction of pTNFAIP3I2 by dex and TNF was due to direct effects of GR and NF-κB we transfected Beas-2B cells with pTNFAIP3I2 and siRNA directed against either GR or the p65 subunit of NF-κB. A

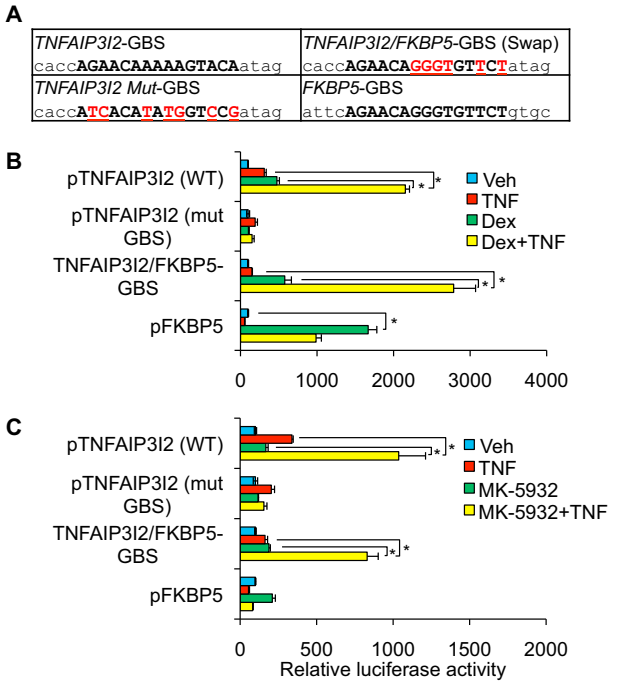

FIGURE 5. **Glucocorticoid receptor binding site context mediates GR/NF-κB synergism.** *A*, sequences of GR binding sites from *TNFAIP3* intron 2, *FKBP5*, and mutations. *B*, relative luciferase activity of the indicated plasmids after transfection into Beas-2B cells and treatment for 8 h with vehicle, TNF (20 ng/ml), dex (100 nM), or TNF + dex. *C*, relative luciferase activity of the indicated plasmids after transfection into Beas-2B cells and treatment for 8 h with vehicle, TNF (20 ng/ml), MK-5932 (100 nM), or TNF + MK-5932. For *B* and *C*, bars represent mean luciferase activity normalized to that of the control reporter (SV40-*Renilla*) + S.D. * indicates $p \leq 0.05$ for relevant comparisons.

reporter with three multimerized NF-κB binding sites, p3XNF-κB, was used as a positive control for NF-κB activity (32), whereas an established GR-responsive reporter for FKBP5 (28), here termed pFKBP5, served as a positive control for GR. Knockdown of p65 eliminated the induction of both p3XNF-κB and pTNFAIP3I2 by TNF. The combinatorial effect of TNF and dex on pTNFAIP3I2 was also reduced to ~35% of levels obtained with control siRNA transfection. Similarly, knockdown of GR prevented the inductive effects of dex on the pTNFAIP3I2 and pFKBP5 reporters and substantially reduced cooperative induction of pTNFAIP3I2 by TNF + dex co-treatment. Western blots confirmed that transfection with siNF-κB and siGR reduced the protein levels of p65 and GR, respectively. Thus, induction of pTNFAIP3I2 by TNF and dex is mediated through NF-κB and GR.

GR Binding Site Context Controls GR/NF-κB Synergism—GR can modulate NF-κB activity both through glucocorticoid binding sites and also in the context of regulatory regions that lack a strong consensus GR binding sequence (21, 33, 34). Therefore, to test whether the GR binding sequence within the second intron of TNFAIP3 was required for GR/NF-κB cooperativity, we disrupted this sequence using site-directed mutagenesis (Fig. 5*A*) and tested the resultant plasmid, pTNFAIP3I2 (mutGBS), for inducibility by dex and TNF. The activity of pTNFAIP3I2 was entirely dependent on the presence of this GBS (Fig. 5*B*). Next, to determine whether specific sequence features of this GBS regulate transcriptional outcome, we replaced the native *TNFAIP3I2* GBS with a distinct GBS from the *FBKP5* locus that is required for regulation of the pFKBP5 reporter by GR (28). This plasmid, pTNFAIP3I2/FKBP5 GBS, exhibited similar induction to the wild type pTNFAIP3I2 plasmid both with dex treatment and after combined treatment with dex and TNF. Moreover, dex-mediated induction of pTNFAIP3/FKBP5 GBS was substantially less than induction of pFKBP5 despite the two plasmids harboring the identical GBS. Taken together, these data indicate that regulation of pTNFAIP3I2 by GR depends on the presence of a single GBS and that the surrounding context of this GBS is a major determinant of the transcriptional response to dex and TNF.

A Selective GR Ligand Distinguishes between GR/NF-κB Cooperation and Activation of a Simple GR Response Element—Selective GR ligands have been developed that alter GR-mediated transcriptional regulation differentially in comparison with classical synthetic GR agonists such as dexamethasone. A design goal for such ligands has been to enable GR-mediated repression of NF-κB function, while reducing GR-mediated gene induction (16). However, it is unknown whether selective ligands enable GR to cooperate with NF-κB to enhance transcription, an activity that our data implicates as regulating the expression of TNFAIP3, a potent anti-inflammatory protein. We therefore tested the ability of one such ligand developed by Merck and Co., MK-5932 (24), to regulate pTNFAIP3I2. Treatment with MK-5932 and TNF resulted in cooperative induction of pTNFAIP3I2 that was ~40% of the level achieved with dex and TNF treatment (Fig. 5). In contrast, pFKBP5 was only weakly induced by MK-5932 to ~10% of the level obtained with dex. These data indicate that MK-5932 selectively maintains cooperation between GR and NF-κB to induce pTNFAIP3I2 in comparison with induction of pFKBP5. Moreover, although pFKBP5 was only weakly induced by MK-5932, combined treatment with MK-5932 and TNF resulted in induction of the pTNFAIP3/FKBP5 swap that was comparable with the effect of MK-5932 + TNF on wild type pTNFAIP3I2 (Fig. 5*C*). Taken together, these data indicate that GR/NF-κB cooperation can be pharmacologically distinguished from classical GR-mediated induction of an isolated GBS. Moreover, the context of the composite GR/NF-κB regulatory element within *TNFAIP3*, rather than the sequence of the GBS, is the major determinant of combinatorial induction by GR and NF-κB.

DISCUSSION

Glucocorticoids are extremely effective anti-inflammatory agents, but the mechanisms that underpin their potency are not fully understood. Here we show that in comparison with antibody-based blockade of TNF, glucocorticoids selectively spare or augment the expression of negative feedback targets of TNF such as *TNFAIP3*, *TNIP1*, and *NFKBIA*. In contrast to well described repression of NF-κB by GR, co-treatment with dex + TNF caused GR and NF-κB to synergistically induce transcriptional responses through an intronic enhancer for *TNFAIP3*. GR/NF-κB cooperation depended on the context of the GR binding site, rather than the specific binding site sequence, and could be dissociated from induction of a simple glucocorticoid response element by GR through altered ligand chemistry. Our data support a model (Fig. 6) in which context-dependent cooperation between GR and NF-κB alters the balance between

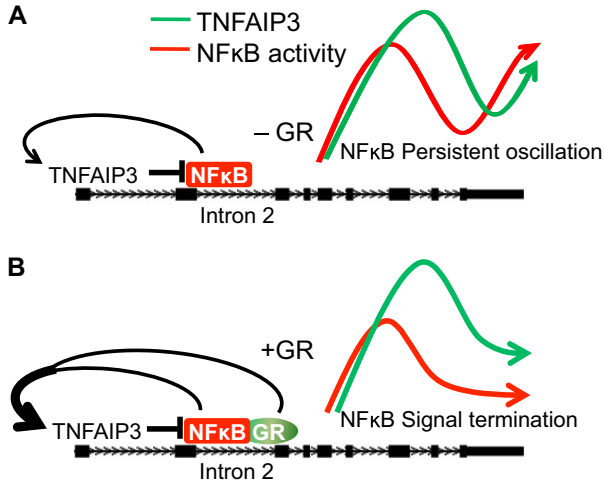

FIGURE 6. **A model of cooperative regulation of *TNFAIP3* by GR and NF-κB leading to termination of NF-κB signaling.** *A*, due to negative feedback regulation of NF-κB, in the absence of activated GR, the level of NF-κB transcriptional activity oscillates (41). As NF-κB drives *TNFAIP3* expression, TNFAIP3 levels oscillate in phase with NF-κB activity levels. *B*, activation of GR signaling maintains *TNFAIP3* expression through cooperating with NF-κB, resulting in a shift of *TNFAIP3* expression levels relative to NF-κB activity. TNFAIP3-mediated reduction of NF-κB activity below a threshold can lead to signal termination.

positive and negative feedback control of inflammation to bias termination of inflammatory responses.

Although GR inhibits numerous NF-κB-regulated genes, two earlier studies described promoters with three NF-κB sites in proximity to a GBS that were co-induced by GR and NF-κB (35, 36). GR occupancy has also been observed to have a neutral effect on NF-κB activity at the *IkB* promoter (31). More recent genome-wide studies in HeLa cells and murine macrophages have indicated that GR and NF-κB co-occupy numerous loci (21, 22) and have associated factor occupancy with both induction and repression of steady state gene expression, including activated GR augmenting the induction of *TNFAIP3* by TNF in HeLa cells. Our study extends on these findings through establishing strongly cooperative enhancement of transcription as a possible outcome of GR/NF-κB co-occupancy. Whether GR/NF-κB cooperation occurs across a range of cell types and encompasses other negative feedback targets of NF-κB remains to be determined.

The cooperative interaction between NF-κB and GR at the *TNFAIP3* enhancer we observed in reporter assays was more than multiplicative. This transcriptional synergism correlated with an ~2-fold increase in endogenous *TNFAIP3* expression after TNF + dex treatment in comparison with treatment with TNF alone. The difference between reporter activity and endogenous *TNFAIP3* expression levels is likely secondary to combinatorial effects of dex and TNF on both positive and negative control of NF-κB, a primary driver of *TNFAIP3* transcription that binds to several sites in the *TNFAIP3* locus. In that regard, as a consequence of negative feedback, NF-κB signaling induces oscillatory cycling of downstream target gene expression that is subject to late-phase damping by TNFAIP3 (37). It remains to be determined experimentally whether the cooperative induction of *TNFAIP3* enhancer activity by GR and NF-κB decouples oscillation of *TNFAIP3* expression relative to NF-κB activity, as we propose in our model (Fig. 6).

The role of GR binding site sequence in determining transcriptional outcomes is currently controversial. Published analyses of the GR cistrome have yielded contradictory conclusions with respect to whether specific GBS sequences mediate transcriptional repression by GR (21, 34). Similarly, although recent studies have indicated that DNA can serve as an allosteric effector that modulates the magnitude of GR-mediated gene induction and influences GR dimerization (9, 38), others have argued that binding site affinity is a primary determinant of GR activity (39). Although our data do not address the role of GBS sequence in transcriptional outcomes on a genome-wide level, they strongly suggest that GR/NF-κB cooperation within the *TNFAIP3* intron 2 enhancer is determined primarily by the context of the binding site, rather than specific GBS sequence features that enable GR/NF-κB cooperativity. Whether the context and positioning of the GR and NF-κB binding sites within the *TNFAIP3* locus result in transcriptional synergism through mediating cooperative binding to DNA by both factors, or through facilitating the engagement of co-activators, has yet to be established.

Dysregulation of *TNFAIP3* transcription is strongly associated with autoimmune disease (13); however, the role of *TNFAIP3* regulation by glucocorticoids in immune-mediated disease pathogenesis and therapeutics has not been established. A recent study demonstrated that LPS-mediated induction of *TNFAIP3* involves looping of both a distal 3′ enhancer and intron 2 to the *TNFAIP3* promoter region (40); looping was disrupted in cells harboring genomic variants associated with the development of lupus. In that system, the recruitment of intron 2 to the *TNFAIP3* promoter was relatively modest, and enhancer properties for intron 2 were not established. Our results raise the possibility that activation of GR signaling will increase LPS-driven association of intron 2 with the *TNFAIP3* promoter/3′ enhancer complex. Long range association of GR-bound intron 2 with other regulatory elements could thus provide a mechanism through which distal SNPs modulate GR responsiveness without directly disrupting a GBS.

Negative feedback is known to play a central role in terminating inflammatory responses and is dysregulated in a number of immune-mediated diseases. Although comparatively little is known about the role of negative feedback in airway inflammation, we propose that cooperation between GR and NF-κB contributes to the efficacy of GR agonists in treating inflammatory airway disorders through augmenting the expression of endogenous anti-inflammatory pathways. A logical extension of this notion is that disruption of GR/NF-κB cooperation may contribute to corticosteroid-resistant airway disease. The availability of clinical samples from patients with airway disease, and murine models in which negative feedback regulators such as *TNFAIP3* are disrupted in the airway, will facilitate future testing of these hypotheses.

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