0026-895X/06/6902-588–596
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:588–596, 2006

Vol. 69, No. 2
19679/3082568
Printed in U.S.A.

CD38 Expression Is Insensitive to Steroid Action in Cells Treated with Tumor Necrosis Factor-α and Interferon-γ by a Mechanism Involving the Up-Regulation of the Glucocorticoid Receptor β Isoform

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Received October 6, 2005; accepted November 16, 2005

ABSTRACT

Evidence shows that the CD38 molecule, recently involved in the two main features of asthma, bronchial hyper-responsiveness and airway inflammation, could represent a new potential therapeutic target for asthma. In this study, we investigated whether glucocorticoid (GC), the most effective treatment for lung diseases, can affect CD38 expression in human airway smooth muscle (ASM) cells treated with different pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interferons (IFNs). We found that CD38 expression induced by TNFα alone was completely abrogated by fluticasone (100 nM), dexamethasone (1 μM), or budesonide (100 nM). In contrast, the synergistic induction of CD38 by the combination of TNFα with IFNγ or IFNβ, but not with IL-1β or IL-13, was completely insensitive to the GC inhibitory effects. We also found that TNFα and IFNγ impaired GC responsiveness by inhibiting steroid induced both 1) GRα-DNA binding activity and 2) GC-responsive element-(GRE)-dependent gene transcription. Although levels of the GC receptor (GR) α isoform remained unchanged, expression of GRβ, the dominant-negative GR isoform, was synergistically increased by TNFα and IFNγ with a GRα/GRβ ratio of 1 to 3. More importantly, fluticasone failed to induce GRE-dependent gene transcription and to suppress TNFα-induced CD38 expression in ASM cells transfected with constitutively active GRβ. We conclude that, upon pro-inflammatory cytokine stimulation, CD38 expression becomes insensitive to GC action by a mechanism involving the up-regulation of GRβ isoform, thus providing a novel in vitro cellular model to dissect GC resistance in primary cells.

CD38 is an ectoenzyme that converts the cellular intermediary metabolite βNAD$^+$ to cyclic ADP ribose (cADPr), a Ca^{2+}-mobilizing second messenger. Previous reports using experimental approaches such as monoclonal agonistic antibodies, the cADPr antagonist 8-bromo-cADPr, or CD38-deficient cells demonstrate a role for CD38 in both B and T cell proliferation, cytokine production from B and T cells (Funaro et al., 1997), neutrophil migration (Deaglio et al., 2003), neurotransmission, and cardiac contraction (Partida-Sanchez et al., 2001). We showed that CD38 pathways also mediate TNFα-enhancing effect on agonist-evoked Ca^{2+} responses (Deshpande et al., 2003) and proposed that abnormal CD38 signaling may represent a novel mechanism involved in the increased airway narrowing observed in asthmatic patients (Amrani et al., 2004; Tliba et al., 2004). We also found that a combination of pro-asthmatic cytokines such as TNFα and IFNs synergistically increased CD38 expression both at protein and mRNA levels (Tliba et al., 2004). Furthermore, in addition to modulating contractile function, we recently reported a putative role of CD38/cADPr pathway in the regulation of different inflammatory genes, such as IL-6 and RANTES, important in the pathogenesis of asthma (Tliba et al., 2004). Together, these observations suggest that abnormal CD38 function and/or expression could play a crit-

This work was supported by grant HL64063 from the National Institutes of Health (to Y.A.), by the Francis Families Foundation (to Y.A.), and by GlaxoSmithKline. Y.A. is a Parker B. Francis Fellow in Pulmonary Research.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.019679.

ABBREVIATIONS: cADPr, cyclic ADP ribose; TNFα, tumor necrosis factor-α; IFN, interferon; IL, interleukin; RANTES, regulated upon activation normal T-cell expressed and secreted; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; ICAM, intercellular adhesion molecule; ASM, airway smooth muscle; PCR, polymerase chain reaction; CA, constitutively active; SEAP, secreted alkaline phosphatase; r, recombinant; COX-2, cyclooxygenase 2; GFP, green fluorescent protein; FP, fluticasone propionate; RT, reverse transcription; CMV, cytomegalovirus.

ical role in two main features of asthma, bronchial hyperresponsiveness and airway inflammation. Thus, a better understanding of both the factors and the mechanisms that regulate CD38 function will provide new therapeutic options for treating airway inflammatory diseases.

Glucocorticoids (GCs) are the treatment of choice for chronic inflammatory diseases such as asthma (Lemanske and Busse, 2003). Most of their anti-inflammatory effects are mediated via glucocorticoid receptor α isoform (GRα), which suppresses expression of inflammatory genes through mechanisms known as transactivation or transrepression (Leung and Bloom, 2003). Transactivation results from a direct binding of activated GRα to DNA sequences called GC-response elements (GRE) present on inducible anti-inflammatory genes. Transrepression is mediated via the direct interaction between activated GRα and different transcription factors, such as nuclear factor κB and activator protein 1, thus repressing transcription factor-inducible pro-inflammatory genes (Pujols et al., 2004). It is noteworthy that in addition to GRα, and as a result of alternative splicing mechanisms, another GR isoform, GRβ, has been described previously (Hollenberg et al., 1985). Although the role of GRβ is not well understood, a previous report in immune and transformed cells demonstrated its dominant-negative effects on GRα-dependent transcriptional activities (Leung et al., 1997).

Increasing evidence suggests that ASM cells play a central role in the pathogenesis of asthma (Parris et al., 1999; Amrani and Panettieri, 2003; Hunter et al., 2003). In fact, when exposed to inflammatory conditions, ASM can become a source of different chemokines/cytokines that are capable of orchestrating and/or perpetuating airway inflammation (Howarth et al., 2004). Although the GC effects on human ASM cells have been investigated, their modulatory effects on gene expression are quite complex, and the signaling mechanisms underlying their suppressive effects have been poorly characterized. First, the effects of GC seem to be highly gene-specific. For example, reports showed that dexamethasone can effectively inhibit cytokine-induced IL-6, RANTES (Ammit et al., 2002), eotaxin (Pang and Knox, 2001), or cyclooxygenase-2 expression (Vlahos and Stewart, 1999), whereas it has no effect on cytokine-induced ICAM-1 expression (Amrani et al., 1999). Second, GC suppressive effect seems to be time-dependent, because dexamethasone partially abrogates cytokine-mediated ICAM-1 expression at early time points but has no effect at later time points (Amrani et al., 1999). Third, unexpectedly, instead of acting as potent inhibitors, GC synergistically enhanced cytokine-induced fractalkine expression and secretion (Sukkar et al., 2004). Finally, GC inhibitory action is also stimuli-specific. Indeed, whereas dexamethasone significantly inhibits granulocyte macrophage-colony stimulating factor secretion induced by IL-1β, it partially inhibits the secretion induced by thrombin. Together, these observations demonstrate that ASM is a unique and complex model in which GC actions are differentially modulated by cytokines.

In the present report, we demonstrate that CD38 expression induced by cytokine combination becomes refractory to the suppressive action of steroid. We found that the specific combination of TNFα with IFNs, but not with IL-1β or IL-13, reduces ASM cell responsiveness to different classes of GC, including fluticasone, dexamethasone, and budesonide. This steroid insensitivity seems to be the result of increased levels of GRβ that impair GRα transcriptional activity.

Materials and Methods

ASM Cell Culture and Treatment. Human tracheal tissue culture was obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. The culture of human ASM cells was performed as described elsewhere (Panettieri et al., 1989).

Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was extracted from human ASM cells using RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. In preliminary experiments, we determined, for each primer pair, the melting temperature and number of amplification cycles necessary to yield the appropriate hybridization signal. The PCR of CD38, GRα, GRβ, and β-actin was performed using previously published primers (Orii et al., 2002; Tliba et al., 2004). Each of 35 cycles of the PCR was programmed to carry out denaturation at 94°C for 30 s, primers annealing at 55°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The semiquantitative PCR approach was performed in parallel by investigating the β-actin mRNA level. The intensity of the area density was analyzed using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD), and the final PCR product was expressed as a ratio of area density of CD38, GRα, or GRβ to β-actin.

Real-Time PCR. Real-time PCR analysis was performed on a Smart Cycler (Cepheid, Sunnyvale, CA) by using a SYBR-Green kit according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). PCR was performed using the same GRα, GRβ, and β-actin primers in a total volume of 25 μl in the presence of SYBR Green PCR Master Mix. For GRα and GRβ PCR amplification, the program consists of 60 s for the initial denaturation period at 95°C, and 45 cycles of 95°C for 1 s, 60°C for 5 s, and 72°C for 15 s. For β-actin, the program consists of 60 s for the initial denaturation period at 95°C, 40 cycles of 95°C for 1 s, 55°C for 5 s, and 72°C for 18 s. The results were calculated using a quantitation approach termed the comparative cycle threshold (C_t) method as described elsewhere (Ponchel et al., 2003).

GR-DNA Binding Activity. Nuclear extraction was performed as described previously (Tliba et al., 2003). Ten micrograms of nuclear extracts were tested for GR-DNA binding activity by using TransAM GR kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). The results (optical densities measured at 450 nm) were expressed as a percentage of increase over untreated cells.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Immunoblot analyses for GRα and GRβ were performed as described previously (Tliba et al., 2003) using specific anti-GRα and anti-GRβ antibodies (Affinity BioReagents, Golden, CO). To ensure equal loading, the membranes were stripped and reprobed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The area densities for each GR isoform and the total actin were calculated using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD), and the data were expressed as a ratio of area density of GRα or GRβ to actin.

Transfection of ASM Cells. Because most standard transfection methods yield poor transfection efficiencies for ASM cells (<10%), here we have optimized a high-efficiency transfection technique. This technique is an extension of electroporation, using Nucleofector kit for primary smooth muscle (Amaxa Biosystems, Cologne, Germany), in which plasmid DNA is transfected directly into the cell nucleus. Transfection was performed according to the manufacturer's instructions, and the program used was D-33. Sixteen to eighteen hours after transfection, the media were changed with serum-free media for the next 24 h. Transfection efficiency of GFP-tagged

plasmids was monitored by flow cytometry as described below. This method, using green fluorescence protein (GFP)-pmax control vector (Amaxa Biosystems), enabled us to reach a transfection efficiency of 70%.

Recombinant Plasmids. To assess the dominant negative activity of GRβ, ASM cells were transfected with constitutively active (CA) GRβ GFP-tagged (Oakley et al., 1996) or with pCMV-GFP empty vector (Clontech, Mountain View, CA). To monitor fluticasone transactivating activity, cells were cotransfected with GRE-secreted alkaline phosphatase (SEAP) reporter vector and with pSV-β-galactosidase vector used to normalize transfection efficiencies (Promega, Madison, WI).

SEAP and β-Galactosidase Assays. The activities of SEAP and β-galactosidase were evaluated using Great EscAPe SEAP detection kit (Clontech) and β-galactosidase detection kit (Promega, Madison, WI), respectively, according to their manufacturer's instructions (Tliba et al., 2003; An et al., 2005).

Fluorescence-Activated Cell Sorting Analysis. Flow cytometry was performed as described previously (Tliba et al., 2002). Antibody used for CD38 expression was purchased from Santa Cruz Biotechnology. Phycoerythrin-conjugated secondary antibody was bought from Jackson ImmunoResearch Laboratories (West Grove, PA). GRβ-GFP transfection efficiency was monitored by a shift to green fluorescence (FL-1), and CD38 expression level was monitored by a shift to red fluorescence (FL-2). Fluorescence-activated cell sorting analysis was performed with EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA) using the Cellquest software.

Materials and Reagents. Tissue culture reagents and primers were obtained from Invitrogen (Carlsbad, CA). Human recombinant (r) TNFα, rIL-1β, and rIFNγ were provided by Roche Diagnostics. Human rIFNβ and rIL-13 were purchased from R&D Systems (Minneapolis, MN). Fluticasone propionate (FP) was generously supplied by GlaxoSmithKline, Inc. (King of Prussia, PA). Dexamethasone and budesonide were obtained from Sigma (St. Louis, MO).

Statistical Analysis. Data points from individual assays represent the mean values of triplicate measurements. Significant differences among groups were assessed with analysis of variance (Bonferroni-Dunn test) or by t-test analysis, with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. Each set of experiments was performed with a minimum of three different human ASM cell lines.

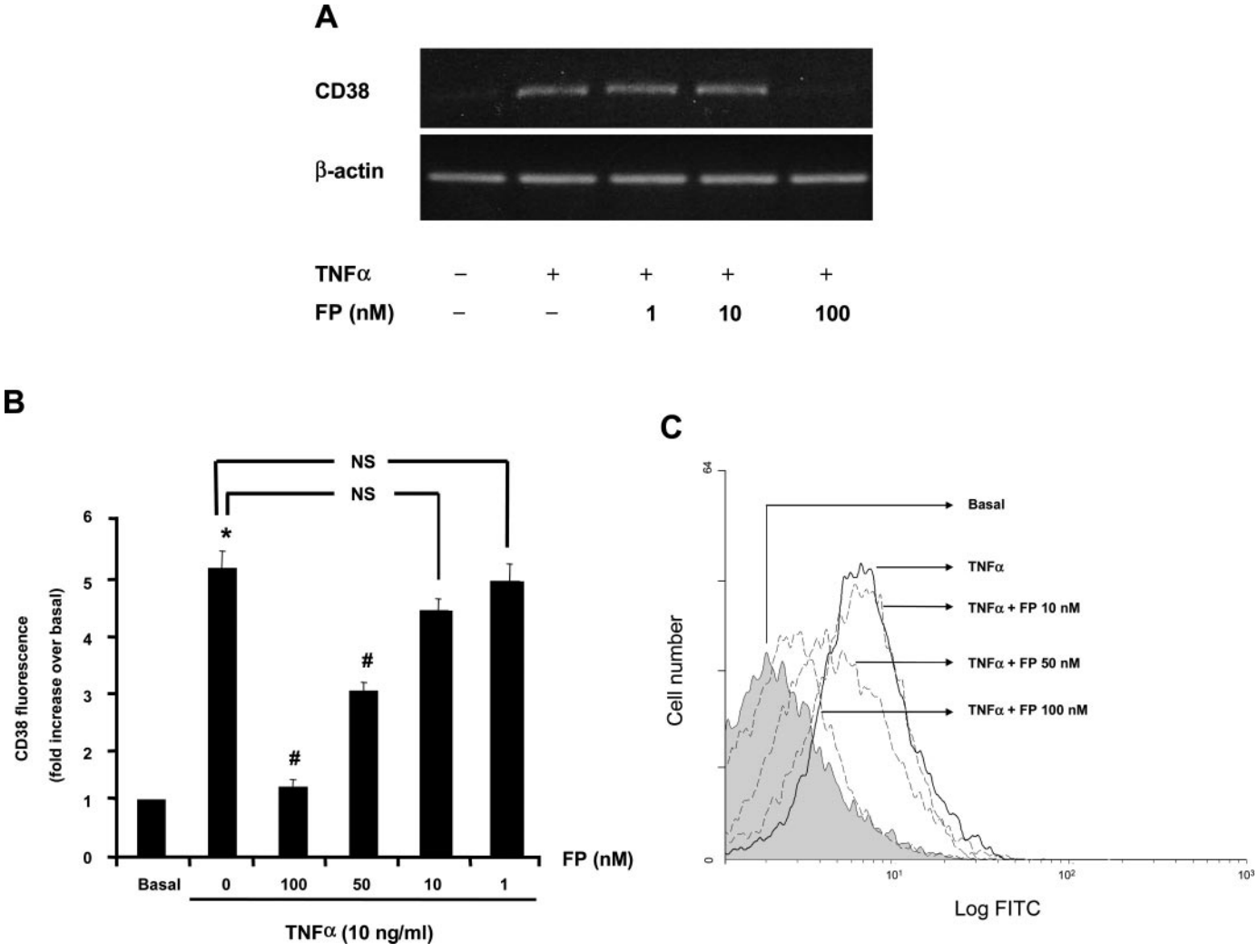

Fig. 1. Dose-dependent effect of fluticasone (FP) on TNFα-induced CD38 expression in ASM cells. Cells were pretreated with indicated concentrations of FP for 2 h and then stimulated with TNFα (10 ng/ml) for 24 h. A, representative agarose gel showing the CD38 PCR products stained with ethidium bromide. Cells stimulated as indicated were lysed, total mRNA was isolated, and RT-PCR was performed as described in the *Materials and Methods* using specific primers for CD38 and β-actin genes. B, CD38 protein expression was assessed by flow cytometry as described in the *Materials and Methods* and the results are expressed as the -fold increase in mean fluorescence intensity over basal (untreated cells) ± S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with TNFα alone; NS, not significant compared with cells treated with TNFα alone. C, representative flow cytometry histograms of GC effect on CD38 expression.

Results

Fluticasone Inhibits in a Dose-Dependent Manner TNFα-Induced CD38 Expression. We first investigated the effect of GC on TNFα-induced CD38 expression, a gene potentially involved in modulating airway inflammation via the transcriptional regulation of different inflammatory mediators (Tliba et al., 2004). For this purpose, human ASM cell cultures were pretreated for 2 h with increasing concentrations of fluticasone (1–100 nM) before TNFα treatment, and CD38 expression was measured by RT-PCR. As shown in Fig. 1A, fluticasone at 100 nM completely inhibited TNFα-induced CD38 mRNA expression ($P < 0.05$). In addition, flow cytometry analyses revealed that fluticasone also inhibited, in a concentration-dependent manner, TNFα-induced CD38 expression ($P < 0.05$) with complete inhibition achieved at 100 nM (CD38 level decreased from 5.2 ± 0.26- to 1.42 ± 0.13-fold increase over basal in cells treated with TNFα in the absence or presence of fluticasone, respectively) (Fig. 1, B and C). In a parallel experiment, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Amrani et al., 1996), we found that fluticasone did not affect ASM cell viability (data not shown). Together, these data suggest that GC suppresses TNFα-induced CD38 expression by involving transcriptional mechanisms.

TNFα Combined with IFNs (β or γ), but Not with IL-1β or IL-13, Dramatically Reduces CD38 Sensitivity to Fluticasone. Because TNFα cooperates with other cytokines to regulate the expression of different inflammatory genes in ASM cells (Tliba et al., 2003), we next investigated whether cytokine combination would induce a greater in-

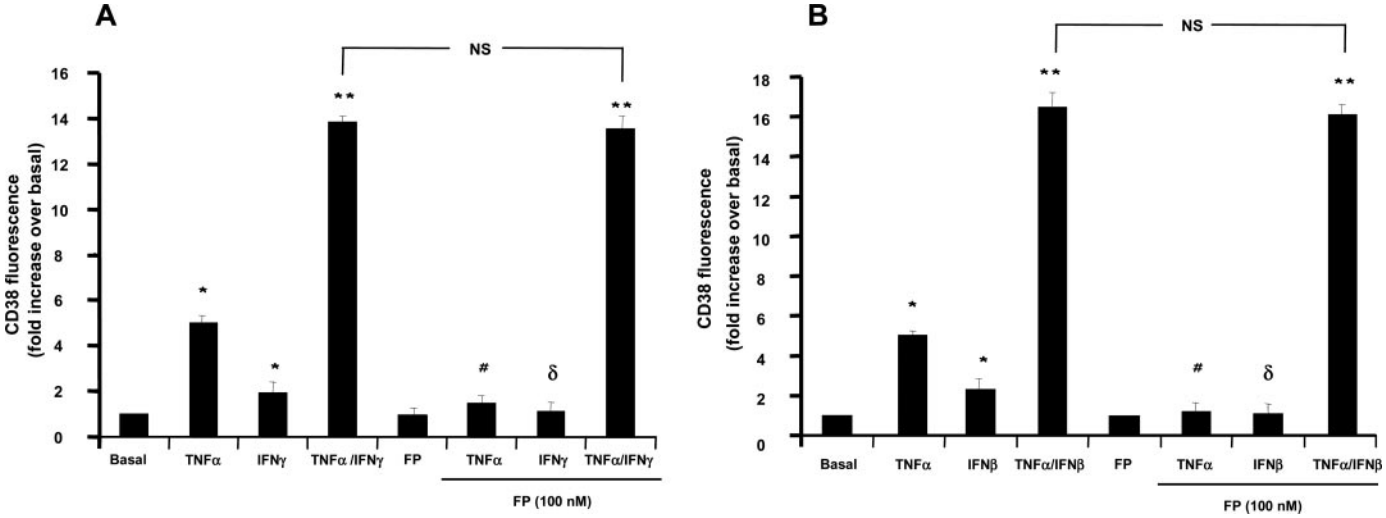

Fig. 2. Effect of TNFα and IFNs combination on CD38 sensitivity to FP. Human ASM cells were treated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) (A) or TNFα (10 ng/ml) and IFNβ (500 IU/ml) (B) for 24 h in the presence or absence of FP (100 nM) added 2 h before. CD38 expression was assessed by flow cytometry as described under *Materials and Methods*. The results are expressed as the -fold increase in mean fluorescence intensity over basal (untreated cells) ± S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells; # and δ, $P < 0.05$ compared with cells treated with TNFα or IFNs alone, respectively; NS, not significant compared with cells treated with TNFα and IFNs combination.

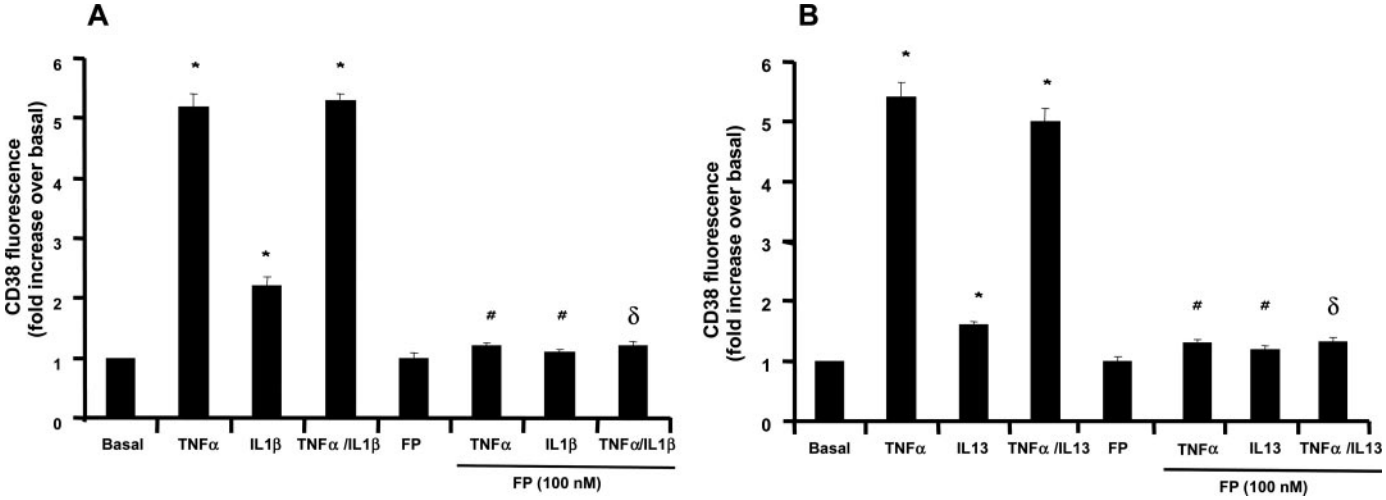

Fig. 3. Effect of TNFα and IL-1β or TNFα and IL-13 combination on CD38 sensitivity to FP. Human ASM cells were treated with TNFα (10 ng/ml) and IL-1β (10 ng/ml) (A) or TNFα (10 ng/ml) and IL-13 (50 ng/ml) (B) for 24 h in the presence or absence of FP (100 nM) added 2 h before. CD38 expression was assessed by flow cytometry as described under *Materials and Methods*. The results are expressed as the -fold increase in mean fluorescence intensity over basal (untreated cells) ± S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; # and δ, $P < 0.05$ compared with cells treated with cytokine alone or in combination, respectively.

crease in CD38 expression and whether this response would be sensitive to steroid. We found that IFNγ (500 IU/ml) (Fig. 2A), IFNβ (500 IU/ml) (Fig. 2B), IL-1β (10 ng/ml) (Fig. 3A), or IL-13 (50 ng/ml) (Fig. 3B) induced a weak but significant increase in CD38 expression with a 2 $\pm$ 0.1-, 2.2 $\pm$ 0.05-, 2.25 $\pm$ 0.12-, and 1.7 $\pm$ 0.09-fold increase over basal, respectively. In addition, whereas IL-1β and IL-13 showed no additive effect when combined with TNFα (Fig. 3, A and B), IFNγ and IFNβ significantly enhanced TNFα-induced CD38 expression by 13.8 $\pm$ 0.15- and 16.2 $\pm$ 0.28-fold over basal, respectively (Fig. 2, A and B). We found that CD38 induction in cells treated with the combination of TNFα with either IFNγ (Fig. 2A) or IFNβ (Fig. 2B) was completely insensitive to effective concentration of fluticasone. However, fluticasone was still effective in preventing CD38 induction in cells treated with the combination of TNFα with either IL-1β (Fig. 3A) or IL-13 (Fig. 3B). Similar results were obtained with other steroids, including dexamethasone (1 μM) or budesonide (100 nM), known to be potent inhibitors of different inflammatory genes in ASM cells (Ammit et al., 2002; Roth et al., 2002) (Fig. 4). Together, these data suggest that TNFα and IFNs is an effective combination 1) to synergistically induce CD38 expression, and 2) to dramatically reduce the effect of different synthetic GCs.

TNFα and IFNγ Together Alter Steroid-Induced Transactivation Activities. The inability of GC to suppress CD38 induced by TNFα and IFNγ (Figs. 2 and 4) may involve an alteration of GC transactivation activities. As

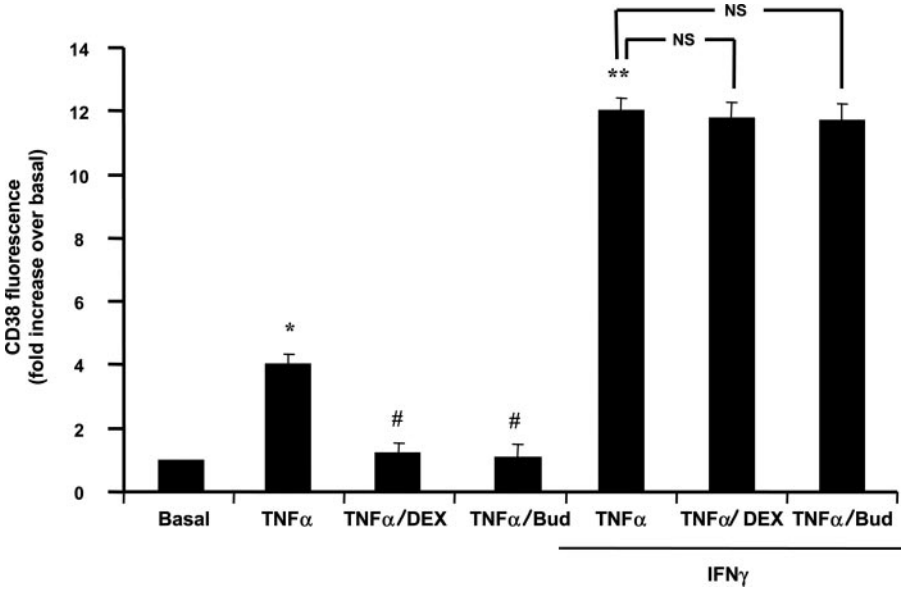

Fig. 4. Effect of TNFα and IFNγ on CD38 sensitivity to dexamethasone and budesonide. Human ASM cells were treated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) for 24 h in the presence or absence of dexamethasone (DEX) (1 μM) or budesonide (BUD) (100 nM) added 2 h before. CD38 expression was assessed by flow cytometry as described under *Materials and Methods*. The results are expressed as the -fold increases in mean fluorescence intensity over basal (untreated cells) $\pm$ S.E.M. of three separate experiments. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with TNFα alone; NS, not significant compared with cells treated with TNFα and IFNγ combination.

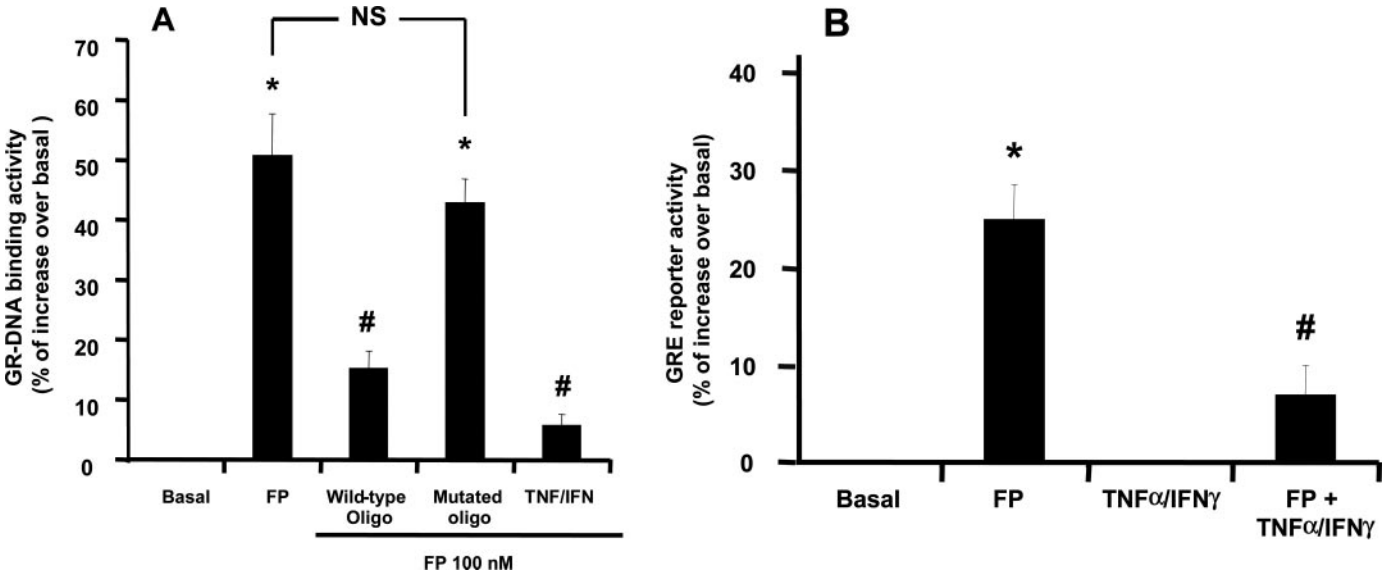

Fig. 5. TNFα and IFNγ combination impairs GC activities. A, ASM cells were treated with fluticasone (100 nM) for 24 h in the presence or absence of wild-type oligonucleotide, mutated oligonucleotide, or TNFα and IFNγ combination. Nuclear extracts were then tested for GR-DNA binding activity using the TransAM GR kit as described in *Materials and Methods*. *, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with fluticasone alone. NS, not significant compared with cells treated with fluticasone alone. B, cells were transfected with 4 μg of SEAP reporter construct containing GRE motifs before fluticasone and cytokines were added. Cells were then lysed, and the SEAP activity was performed as described in *Materials and Methods*. *, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with fluticasone alone.

shown in Fig. 5A, fluticasone significantly enhanced GR-DNA binding activity by 51 ± 5.2% compared with untreated cells, an effect that was completely abrogated when an excess of wild-type competitor oligonucleotides, but not mutated control oligonucleotides, was added. It is noteworthy that, as seen with wild-type oligonucleotides, TNFα and IFNγ completely prevented fluticasone-induced GR-DNA binding activity. Furthermore, in ASM cells transfected with a reporter construct containing SEAP gene driven by GRE motifs, we found that fluticasone significantly enhanced SEAP activity by 25 ± 3.1% compared with untreated cells, a response that was dramatically reduced by 70% when TNFα and IFNγ were added (Fig. 5B). These data suggest that TNFα and IFNγ together impair GC cellular response by reducing both GR-DNA interaction and GR transactivating activity.

TNFα and IFNγ Enhance Selectively the Expression of GRβ Isoform. Evidence suggests that changing the GRα/GRβ cellular ratio in different inflammatory conditions, where GRβ predominates, may promote GC resistance (Pujols et al., 2004). Therefore, we next investigated whether cytokine combination could alter GRα/GRβ ratio in human ASM cells. Using RT-PCR analyses, we found that the steady state of GRα mRNA levels, which is constitutively expressed in untreated cells, was not affected by TNFα or IFNγ either alone or in combination (Fig. 6A). Similar results were obtained by real-time PCR (data not shown). Even though TNFα or IFNγ alone have only a slight stimulatory effect on GRβ levels, their combination significantly increased by 470% the steady state of GRβ mRNA levels (Fig. 7A), a result that was also confirmed by real-time PCR (Fig. 7B). Using immunoblot analyses, we found that although the constitutive expression of GRα protein was not affected by TNFα or IFNs (β or γ), either alone or in combination (Fig. 6B), GRβ protein expression was significantly increased by TNFα and IFNs combination (Fig. 7, C and D). Semiquantitation analyses of GR isoform expression showed that GRα/GRβ ratio was 8:1 in untreated cells, 1:1 in TNFα-, IFNβ-, or IFNγ-treated cells, and 1:2 and 1:3 in TNFα/IFNβ- and TNFα/IFNγ-treated cells, respectively. Together, these data demonstrate that in cells exposed to cytokine combination, GRβ becomes the predominant GR isoform.

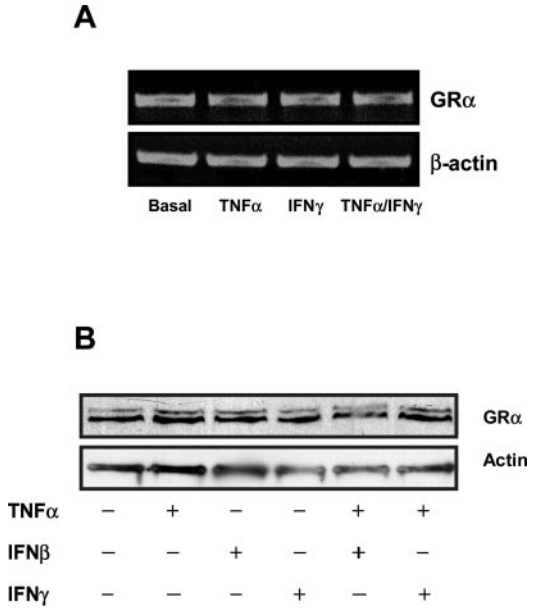

Fig. 6. Effect of TNFα and IFNγ combination on GRα expression. ASM cells were stimulated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) either alone or in combination for 24 h. A, total mRNA (2 μg) was subjected to RT-PCR with β-actin and GRα primers. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. B, in separate experiments, total cell lysates were prepared and assayed for GRα by immunoblot analysis.

GRβ Overexpression Prevents GC-Mediated Transactivation and Reduces the Sensitivity of CD38 to Fluticasone. To further support the association between the reduced action of GC (Figs. 2, 4, and 5) and the enhancement of GRβ expression (Fig. 7) in cells treated with cytokine combination, we next examined the effect of GRβ overexpression on GC activities. To determine whether GRβ interferes with GC-induced transactivation activity, ASM cells were cotransfected with 2 μg of CA-GRβ or pCMV empty vector, and 2 μg of SEAP-reporter construct containing GRE motifs. As shown in Fig. 8A, fluticasone-induced GRE-dependent reporter activity was completely abrogated in GRβ-transfected but not in pCMV-transfected cells. To test whether GRβ interferes with GC actions on CD38 expression, ASM cells were transfected with 4 μg of GFP-tagged CA-GRβ then treated with TNFα for 24 h in the presence or absence of fluticasone, and CD38 expression in GRβ-transfected cells was assessed by two-color flow cytometry. As shown in Fig. 8B, TNFα significantly enhanced CD38 expression in GRβ-transfected cells (35.1 ± 2.2-fold increase over basal), a response that was completely insensitive to fluticasone action (filled bars). However, fluticasone was still effective in inhibiting TNFα-induced CD38 expression in cells transfected with control vector (pCMV-GFP) (open bars). Together, these data suggest that GRβ up-regulation is associated with a significant reduction of GC activities in ASM cells.

Discussion

We and others showed that TNFα and exogenous IFNs synergistically regulate expression of a variety of pro-inflammatory genes, including cytokines, chemokines, and growth factors in ASM cells (Chung, 2000; Tliba et al., 2004). Our latest studies also showed that CD38, an ectoenzyme recently involved in asthma (Deshpande et al., 2003; Tliba et al., 2004), was also responsive to the synergistic action of TNFα and IFNs in ASM cells (Tliba et al., 2004). In the present study, we report that the induction of CD38 induced by both TNFα and IFNs, but not by cytokine alone, is refractory to the anti-inflammatory action of steroids. To our knowledge, this is the first report showing that expression of CD38 can become refractory to the suppressive action of synthetic GC in a clinically relevant cell.

Our data show the TNFα and IFNs render ASM cells insensitive to GC as well as reduce GC-induced transactivation activities. This is an important finding, in that numerous studies suggest a strong association between GC insensitivity and a variety of inflammatory diseases, such as asthma, nasal polyps, and inflammatory bowel disease (Adcock et al., 1995; Honda et al., 2000; Farrell and Kelleher,

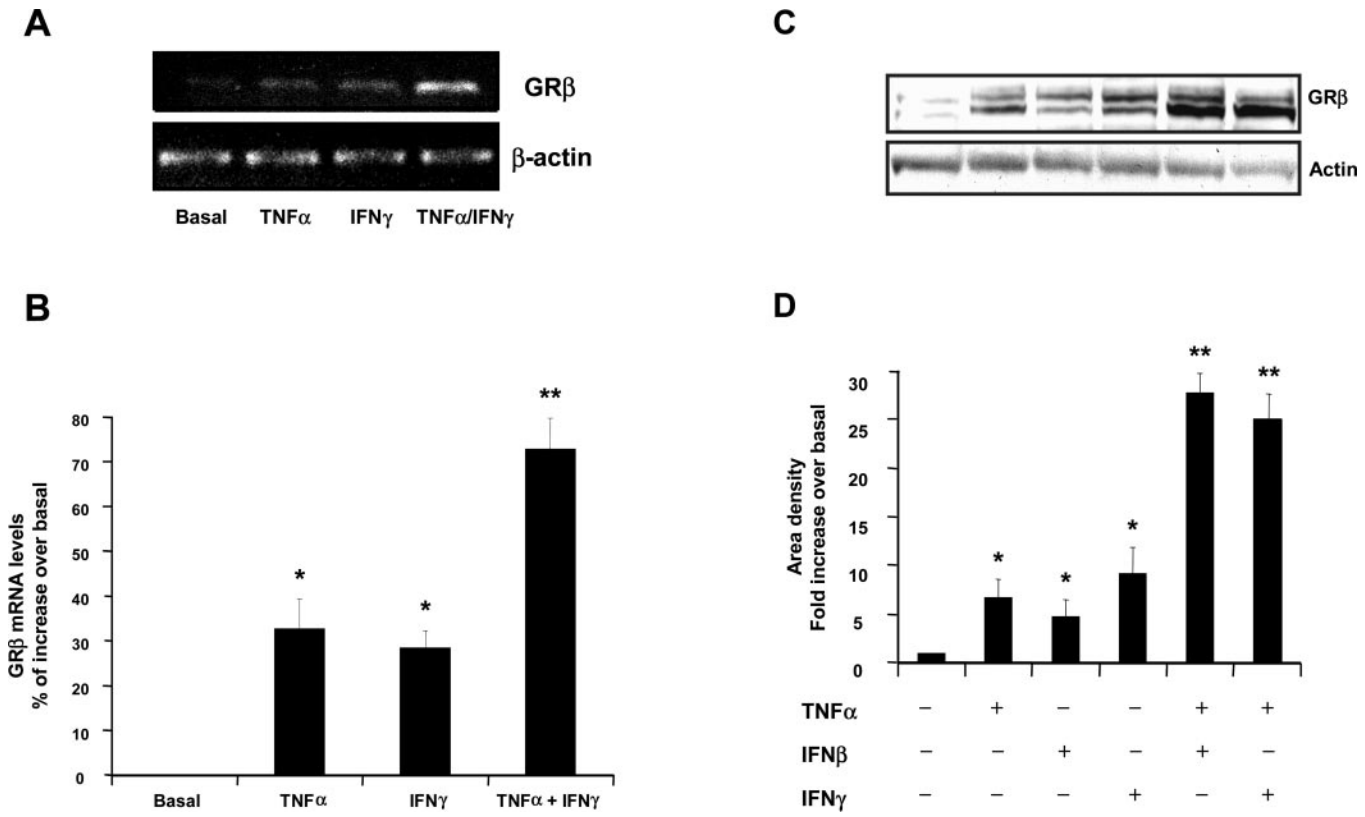

Fig. 7. Effect of TNFα and IFNγ combination on GRβ expression. ASM cells were stimulated with TNFα (10 ng/ml) and IFNγ (500 IU/ml) either alone or in combination for 24 h. A, total mRNA (2 μg) was subjected to RT-PCR with β-actin and GRβ primers. PCR products were separated on a 1% agarose gel and stained with ethidium bromide. B, real-time PCR analysis of GRβ gene. GRβ mRNA levels were normalized to the corresponding levels of β-actin mRNA using the comparative cycle threshold (C_t) method as described under *Materials and Methods*. The results are expressed as a percentage of untreated control values. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells. C, total cell lysates were prepared and assayed for GRβ by immunoblot analysis. D, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding actin content. The results are expressed as the -fold increase over basal. *, $P < 0.05$ compared with untreated cells; **, $P < 0.01$ compared with untreated cells.

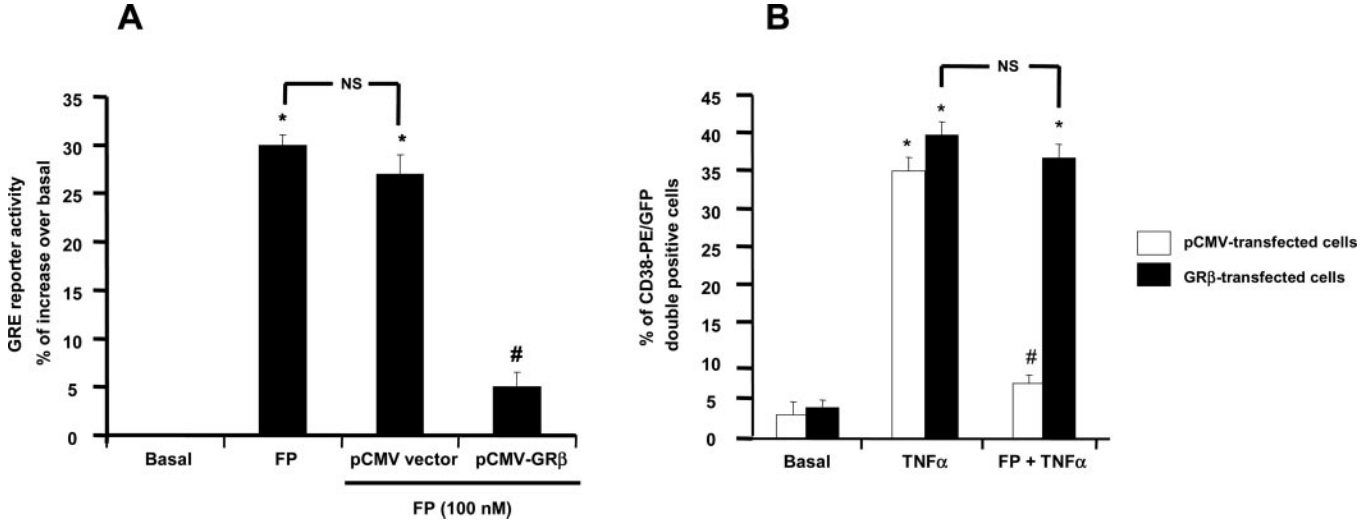

Fig. 8. Effect of GRβ over-expression on fluticasone activities. A, cells were cotransfected with a 2 μg of pCMV empty vector or CA-GRβ construct and 2 μg of SEAP reporter plasmid containing GRE motifs before FP (100 nM) was added for 24 h. SEAP activity was measured as described in the *Materials and Methods*. The results are expressed as a percentage of control values from untreated cells. Data are representative of three separate experiments. *, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with FP alone; NS, not significant compared with cells treated with fluticasone alone. B, flow cytometry analysis of CD38 expression in GRβ-transfected cells. Cells were transfected with 4 μg of pCMV-empty vector or CA-GRβ construct before FP (100 nM) and TNFα (10 ng/ml) were added for 24 h. Cells were then harvested by trypsinization and fixed, and two-color flow cytometry was performed to assess CD38 expression on GRβ-positive cells as described under *Materials and Methods*. The percentage of double-positive cells [i.e., pCMV-GFP/CD38 (open bars) or GRβ-GFP/CD38 (filled bars)] was then calculated and presented in bar graph. *, $P < 0.05$ compared with untreated cells; #, $P < 0.05$ compared with cells treated with TNFα alone; NS, not significant compared with cells treated with TNFα alone.

2003). These observations prompted investigators to examine whether inflammatory mediators could be responsible for the altered GC cellular responses. Impairment of GC activity can be reproduced in vitro in immune cells exposed to IL-2 and IL-4 that reduced the inhibitory effect of methylprednisolone on mitogen-induced T cell proliferation (Leung and Bloom, 2003). Other studies in human peripheral blood mononuclear cells showed that the combination of IL-1β, IL-6, and IFNγ also decreased the suppressive action of dexamethasone on mitogen-induced proliferation (Almawi et al., 1991). In T cells, monocytes, and neutrophils, GC action is as well impaired when exposed to a single cytokine such as IL-2, IL-7, IL-13, IL-15, or IL-8 (Spahn et al., 1996; Pipitone et al., 2001; Strickland et al., 2001; Goleva et al., 2002). Although all in vitro studies describing GC insensitivity have been performed in either immune or transformed cells, very little is known about the modulation of GC signaling in structural cells that are relevant for lung diseases. We now show that the specific combination of TNFα with IFNs reduces ASM cell responsiveness to different classes of GC, including fluticasone, dexamethasone, and budesonide (Figs. 2 and 4). This TNFα- and IFNγ-dependent GC insensitivity was not observed when TNFα was combined with other cytokines, such as IL-1β or IL-13 (Fig. 3), although these latter cytokines significantly reduced GC cellular response in other cell types (Spahn et al., 1996; Pariante et al., 1999; Webster et al., 2001). Our findings could explain, at least in part, the lack of dexamethasone to suppress fractalkine secretion in ASM cells treated similarly with both TNFα and IFNγ (Sukkar et al., 2004). These data suggest that TNFα and IFNs is a new cytokine combination inducing GC insensitivity in human ASM cells, thus providing a novel in vitro model to dissect GC insensitivity in structural cells. The mechanisms underlying TNFα/IFNs suppressive effects on GC signaling and function are unknown, but our study demonstrates the potential role of GRβ.

The fact that the inhibitory effect of cytokine combination on GC actions could be mimicked in cells transfected with CA-GRβ (Fig. 8) strongly suggests that GRβ represents one molecular mechanism responsible for the impaired GC activity. We found that the combination of TNFα and IFNs significantly increased GRβ isoform at both mRNA and protein levels but had a weak effect on GRα expression. In peripheral blood mononuclear cells, where the levels of GRβ were significantly increased by IL-7 or IL-18, no changes in GRα levels were observed (Orii et al., 2002). In neutrophils, however, IL-8 enhanced GRβ expression, whereas the GRα expression was decreased (Strickland et al., 2001). Moreover, Torrego et al. (2004) showed that IL-2 and IL-4 combination had no effect on GRβ expression but enhanced significantly GRα expression contrasting with the selective induction of GRβ by the same cytokine combination observed by Leung et al. (1997). Together, these observations suggest that GR isoforms are differentially regulated in a complex stimuli- and cell-specific manner. The important question that remains unanswered is the nature of the specific mechanisms that regulate the selective induction of GRβ. Because GRβ expression is a result of alternative splicing (Hollenberg et al., 1985), it is plausible that the selective GRβ induction by TNFα and IFNs occurs because of the possible activation of specific alternative splicing factors. Indeed, Xu et al. (2003) showed that increased levels of the serine-arginine–rich protein SRp30 is required for the alternative splicing of GR pre-mRNA to GRβ isoform in neutrophils. In our study, cytokine combination failed to increase SRp30 content in ASM cells (O. Tliba and Y. Amrani, unpublished observations). Further investigations are needed to determine the alternative splicing factors that are involved in the differential induction of GR isoforms in ASM cells.

So far, the inhibitory role of GRβ on GRα activity remains controversial. Oakley et al. (1999) found that the transient transfection of increasing amounts of GRβ-expressing vectors into immune and transformed cells inhibits its GC actions by blocking the transactivating ability of GRα. In contrast, Hecht et al. (1997) and de Lange et al. (1999) were unable to confirm the inhibitory role of GRβ when overexpressed into COS-1 cells. These controversial results may be explained by the use of different experimental conditions, such as the transfection methods, the promoter used to overexpress GRβ, and the relative amounts of transfected plasmids, which could lead to insufficient GRβ expression. Indeed, a high expression level of GRβ isoform is necessary to significantly inhibit GC-mediated gene expression in COS-1 cells (Oakley et al., 1999). This hypothesis is supported in our study by the fact that in cells exposed to cytokine alone (TNFα or IFNγ), where GRβ expression was slightly enhanced, reaching a level comparable with that of GRα (Figs. 6 and 7), fluticasone was still able to suppress cytokine-induced CD38 expression (Fig. 1). In contrast, we showed that in cells resistant to fluticasone action [i.e., treated with both TNF/IFNs (Fig. 2)], GRβ level was significantly predominant over that of GRα (ratio of GRα to GRβ was $\sim$1:3). The dominant-negative properties of GRβ were difficult to demonstrate in primary cells because most standard transfection methods yield poor transfection efficiencies ($<$10%). To overcome the low transfection efficiencies, we have optimized a high-efficiency transfection technique. This technique is an extension of electroporation, using a Nucleofector kit for primary smooth muscle (Amaxa Biosystems). Using this method, we showed that the GC ability to block TNFα-induced CD38 expression as well as to induce GRE-dependent gene transcription was completely impaired in ASM cells transfected with CA-GRβ (Fig. 8). To our knowledge, this is the first study in primary cells showing that proasthmatic cytokines alter steroid function via the induction of GRβ.

In summary, we found that the combination of TNFα and IFNs renders CD38 expression refractory to the suppressive action of steroid by a mechanism involving the GRβ-dependent alteration of GRα signaling and function. The importance of IFNs in promoting GC insensitivity in airway resident cells could explain, at least in part, the increased GC requirements in patients with severe asthma experiencing viral infections that produce high levels of IFNs in the airways (Johnston, 1999; Yamada et al., 2000). Our study opens a new area of investigation to determine the molecular mechanisms by which cytokine-induced GRβ expression impairs GC signaling in structural cells.

Acknowledgments

We thank Mary McNichol for assistance in the preparation of the manuscript.

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